PREGNANCY RECOGNITION IN CATTLE: EFFECTS OF CONCEPTUS PRODUCTS ON UTERINE PROSTAGLANDIN PRODUCTION

BY

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Chairman: William W. Thatcher
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In cattle, continued progesterone (P_4) production by the corpus luteum (CL) is required if pregnancy is to persist. Identification of putative conceptus-derived signals and evaluation of their biological roles relative to CL maintenance during early pregnancy were goals of this research endeavor.

Using various chromatography systems, bovine conceptuses (days 19 to 23) exhibited extensive metabolism of tritiated P_4 (90-98%), in vitro. A majority of conceptus metabolites were 5β-reduced pregnanes. A major conceptus metabolite was 5β-pregnan-3α-ol-20-one (5β-P). Conversely, endometrial explant cultures metabolized 40 to 50% of P_4 substrate to primarily 5α-reduced steroid products.

An in vivo test system to evaluate uterine $PGF_{2\alpha}$ production capacity was characterized in experiment two. Exogenous estradiol-17β (E_2; 3 mg I.V.) stimulated uterine blood flow, and $PGF_{2\alpha}$ production and metabolism. Concentrations of the primary metabolite of $PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM) were significantly correlated (r=.66) with the E_2-induced uterine $PGF_{2\alpha}$ production response. Use of peripheral PGFM concentrations as an index of uterine $PGF_{2\alpha}$ production was supported.

In experiment three, CL function, interestrous interval, and spontaneous uterine $PGF_{2\alpha}$ production were evaluated in cyclic cows following intrauterine administration of 5β-P, conceptus secretory proteins (CSP) or homologous serum proteins (Control). Extensions in CL lifespan and interestrous interval were detected in cows administered CSP compared to 5β-P and Control group responses. Lifespan of CL and estrous cycle lengths were not different (P>.25) between 5β-P and Control groups. Spontaneous $PGF_{2\alpha}$ episodes were depressed in CSP-treated cows but not in cows administered 5β-P or serum proteins. In experiment four, E_2-induced uterine $PGF_{2\alpha}$ production (peripheral PGFM response) was evaluated in cyclic cattle following interuterine administration of bovine CSP or day 18 pregnant serum proteins (Control). Mean concentrations of PGFM in CSP-treated cows were depressed (P<.01) compared to Control responses. Estradiol injection failed to elicit any PGFM

response in three of five CSP-treated cows. In contrast, all Control animals exhibited PGFM responses during the period of E_2-induced $PGF_{2\alpha}$ production. Results support a role for CSP in suppression of uterine $PGF_{2\alpha}$ production during early pregnancy in cattle.

CHAPTER 1
REVIEW OF LITERATURE

Introduction

The recurrent nature of estrous cycles in cattle centers around development and regression of the ovarian corpus luteum (CL). Progesterone (P_4), the primary product of CL, directs uterine development and secretory activity such that an embryotropic uterine environment is established during each estrous cycle. Luteal maintenance and P_4 production are essential if successful pregnancy is to be established. The process of CL maintenance during early pregnancy involves numerous and complex biochemical interactions between the conceptus and its maternal host. Our evaluation of mechanisms by which the conceptus and maternal units interact during early pregnancy depend upon identification of putative conceptus-derived 'signals' and development of biological test systems through which conceptus product function may be assessed. It was in this light that research described herein was conducted.

The Bovine Estrous Cycle

General Considerations

The nature of the estrous cycle has been characterized extensively in cattle. Early observations and documentation of the recurrence of sexual behavior in animals provided the first clue as to the cyclical nature of reproductive processes in the female (Heape, 1900; Marshall, 1922; Hammond, 1927; Asdell, 1969). Wallace in 1876 and Ellenberger in 1892 (Hammond, 1927; as cited in Marshall, 1922) were among the earliest to report accurately the interval between "sexual periods" in domestic female cattle.

The cyclical recurrence of estrous behavior follows a periodicity of approximately 21 days with a range of 17 to 25 days considered normal (Hammond, 1922; Asdell et al., 1949; Olds and Seath, 1951; Cupps et al., 1969). Nulliparous heifers tend to exhibit slightly shorter (approximately 1 day) interestrous intervals than multiparous cows (Hammond, 1927; Olds and Seath, 1951).

Unlike sheep (Goodman and Karsch, 1981) and horses (Sharp, 1980), photoperiod does not influence consistently annual reproductive patterns in mature cattle (Rzepkowski et al., 1982). Thus, reproductive activities in domestic cattle are not restricted to specific seasons of the year (Heape, 1900; Hammond, 1927). However, attainment of

puberty in cattle is enhanced by increased daily exposure to light (Hansen et al., 1983).

Loss of body condition due to decreased nutrient availability, lactational stress and parasitism has detrimental effects on cyclicity and reproductive efficiency in cattle (Lamond and Bindon, 1969; Dunn et al., 1969; Rakha and Igboeli, 1971). Thus, wild cattle are known to exhibit limited breeding and calving seasons under natural conditions (Marshall, 1922; Heape, 1900). When such stresses are reduced via management schemes in the confines of a zoo, wild cattle are capable of breeding at all times of the year (Heape, 1900).

High ambient temperature and humidity are other important environmental influences which reduce expression of estrus, blood flow to the reproductive tract, and may alter the normal endocrine milieu in cattle (Thatcher and Collier, 1985). Consequently, heat stress results in decreased reproductive efficiency.

Historically, observations regarding cyclical alterations in behavior and ovarian morphology led Walter Heape (1900) to propose terminology which established the first classification of these cyclical changes into four recurrent phases. Thus, the estrous cycle ("diestrus cycle" by Heape's terminology) in cattle consists of four phases: proestrus (day 19 to estrus), estrus (day 0), metestrus (day 1-3) and diestrus (day 4-18).

Physiology and Endocrinology

Sexual receptivity of the female, or estrus, is a convenient behavioral landmark which is commonly employed to mark the start of an estrous cycle (day 0). Estrus is often characterized by increased restlessness (Kiddy, 1976), vocalizations, mounting activity and, most notably, standing to be mounted by other cattle (Howes et al., 1960). Other variables related to estrus are discussed by Lewis and Newman (1984). Average length of behavioral estrus approaches 18 to 20 hours in mature cattle (Trimberger and Hansel, 1955; Schams et al., 1977); however, cattle in subtropical regions generally experience shorter (10 to 13 hour) phases of estrus (Branton et al., 1957; Chenault et al., 1975).

The temporal sequence of events which culminate in behavioral estrus, and later in the preovulatory gonadotropin surge, involve a synchronized and interdependent cascade of physiological changes in the central nervous system, hypothalamus, pituitary, ovaries and uterus. Characterization of endocrine profiles and study of mechanisms controlling their changes have, at least partially, unravelled the dynamics of various reproductive processes.

Approximately 2 to 4 days before behavioral estrus and the preovulatory surge of gonadotropins, regression of the corpus luteum (CL) is initiated, as determined by a precipitous decline in plasma progesterone (P_4)

concentrations (Chenault et al., 1975; Peterson et al., 1975). In association with this decrease in P_4, pulse frequencies of LH (Rahe et al., 1980; Schallenberger et al., 1984) and FSH (Schallenberger et al., 1984) increase, as do basal concentrations of LH, but not FSH (Chenault et al., 1975; Roche and Ireland, 1981; Milvae and Hansel, 1983b; Schallenberger et al., 1984).

Plasma concentrations of estradiol-17β (E_2) begin to rise during this period (Chenault et al., 1975; Walters and Schallenberger, 1984; Schallenberger et al., 1984) as a result of the initiation and accelerated growth of the preovulatory follicle (Dufour et al., 1972; Merz et al., 1981; Staigmiller et al., 1982; Ireland and Roche, 1982; McNatty et al., 1984a,b). In fact, approximately ten times more E_2 was measured in ovarian vein plasma draining the ovary containing the preovulatory follicle than the contralateral ovary at estrus in cattle (McNatty et al., 1984a).

A significant correlation exists between LH and E_2 pulse frequencies during all phases of the bovine estrous cycle (Schallenberger et al., 1984; Walters and Schallenberger, 1984; Walters et al., 1984). Prior to the gonadotropin surge, LH pulsatile episodes occur every 30 minutes (high frequency) with E_2 pulses being generated responsively (Walters and Schallenberger, 1984). Additionally, E_2 pulse amplitudes gradually increase as estrus approaches. However, these alterations in E_2 pulse

amplitude are not reflected by similar amplitude elevations in LH episodes (Walters and Schallenberger, 1984). This may suggest that LH pulse frequency and follicle sensitivity to LH are major regulatory factors for follicular E_2 production in the cow.

Before reviewing data in support of such a claim, a brief description of follicular anatomy and the two-cell mechanism of follicular estrogen biosynthesis is in order.

The ovarian follicle consists of two steroidogenically active cellular compartments: the vascularized theca interna and the avascular granulosa, which is separated from the theca interna by an acellular basal laminae (see: McNatty et al., 1984b, for references). According to the two-cell mechanism of follicular estrogen production proposed by Falck (1959), the theca compartment metabolizes C-21 steroids to androgens which are then utilized by the granulosa compartment to synthesize estrogens. More recent evaluation of steroidogenic pathways utilized by bovine follicular compartments (Lacroix et al., 1974) support this concept. The theca cell layer utilizes, almost exclusively, the Δ^5-pathway (pregnenolone and 17α-hydroxy-pregnenolone) to synthesize androgens, of which androstenedione (A_4) is the major product. In vivo evaluation of A_4 concentrations in ovarian vein plasma and A_4 production by the ovary throughout the estrous cycle in cattle (Wise et al., 1982) agree with in vitro appraisals of thecal androgen

biosynthesis by Lacroix and coworkers (1974). Metabolism of A_4 to estrogens by the granulosa is very efficient. Conversely, only small amounts of estrogen are synthesized by the theca compartment. Lacroix and coworkers (1974) observed greater yields of estrogen with combined incubations of theca and granulosa cells than yields with incubations of either cell type separately, indicative of a synergism between the two follicle compartments (Falck, 1959).

The capacity of follicles to synthesize estrogens depends upon their ability to respond to gonadotropins. In the cow, 85% of large ($\geq$ 8 mm diameter) follicles on both ovaries bound FSH to granulosa cells and hCG (LH) to theca interna cells, while only 38% of these follicles also bound hCG to granulosa. Follicles with granulosa hCG binding sites were associated with enhanced E_2 concentrations in follicular fluid (England et al., 1981; Merz et al., 1981). Sheep also exhibit a high positive correlation between hCG binding to granulosa and follicular E_2 content and production (Webb and England, 1979, 1982). McNatty et al. (1984a) suggested that there was at least one follicle greater than 5 mm (range 1 to 3 follicles per cow) with granulosa cells possessing aromatase activity on day -5 through estrus. However, most, if not all, healthy bovine follicles greater than 2 mm and many atretic follicles were capable of secreting A_4 in response to LH. In no cases were

follicles with granulosa aromatase activity found in the absence of an LH-responsive thecal compartment. Likewise, Bartol et al. (1981) demonstrated that large follicles capable of significant E_2 production were present in ovaries of cattle throughout the estrous cycle.

In the rat (Nimrod et al., 1977), pig (Channing, 1975) and sheep (Weiss et al., 1978) ovarian follicle, granulosa binding sites for hCG/LH are induced by FSH, thereby enhancing granulosa cell responsiveness to LH. Additionally, P_4, dihydroxytestosterone (DHT) and E_2 plus FSH synergistically stimulated granulosa cell responsiveness to LH above levels found with FSH alone (Rani et al., 1981). Low concentrations of E_2 enhance P_4 production by isolated bovine theca cells (Fortune and Hansel, 1979), suggesting that E_2 may also regulate theca sensitivity to LH.

Thus, follicular development of granulosa aromatase activity appears to occur secondarily to the thecal capacity to synthesize androgens. Androgen biosynthesis by the theca interna compartment is enhanced by exposure to LH, while FSH may be important in sensitization of granulosa cells to LH. Steroids, such as P_4, DHT and E_2 may also potentiate this FSH effect.

One apparent consequence of granulosa-binding of LH is activation of the aromatase enzyme complex. Mechanisms involved in the selection of follicles which become

LH-responsive probably depend upon factors other than FSH alone, perhaps intraovarian factors (Alexander et al., 1978; Darga and Reichert, 1978; Schomberg, 1979; Hsueh et al., 1983; Hansel and Convey, 1983), since granulosa cells in a majority of bovine follicles appear to bind FSH, yet in only one to three large follicles do granulosa cells develop the ability to bind LH and acquire aromatase activity (Merz et al., 1981; Bartol et al., 1981; McNatty et al., 1984).

As mentioned, steroidogenic capacity of ovarian follicular compartments depend upon their ability to respond to gonadotropins. Additionally, periodicity and magnitude of gonadotropin release from the anterior pituitary play important roles in ovarian function. Gonadotropin patterns are, in turn, regulated by gonadal steroids via long loop feedback mechanisms. In ovariectomized sheep (Goodman and Karsch, 1980), frequency of tonic LH episodes is decreased by P_4 with no apparent effect on pulse amplitude, while E_2 reduces LH pulse amplitude and does not influence frequency of LH release. Similar observations were made by Rahe et al. (1980) following characterization of LH episodes during mid-luteal and follicular phases of the estrous cycle in intact cows. High amplitude, low frequency LH episodes which occur during P_4-dominated phases of the cycle are thought to result from P_4 negative feedback on the central nervous system's (CNS) oscillator regulating episodic secretion of gonadotropin releasing hormone (GnRH) (Knobil,

1980) and pituitary sensitivity to GnRH (Padmanabhan et al., 1982). During estrogen-dominated phases, prior to the preovulatory surge of gonadotropins, E_2 initially decreases pituitary sensitivity to GnRH resulting in less LH released per GnRH pulse (Kesner et al., 1981; Kesner and Convey, 1982). However, by approximately 8 hours before the gonadotropin surge, when highest preovulatory plasma concentrations of E_2 occur (Walters and Schallenberger, 1984), pituitary sensitivity to GnRH reaches its maximum (Kesner et al., 1981; Kesner and Convey, 1982; Padmanabhan et al., 1982). Yet during this period, amplitude (but not frequency) of gonadotropin episodes remains low (Walters and Schallenberger, 1984). Walters and Schallenberger (1984) suggested that a negative feedback effect of the elevated E_2 on the hypothalamus may explain these endocrine patterns during the preovulatory period.

Regulation of FSH secretion by gonadal steroids appears to be more subtle than observed for LH. Concentrations of FSH are considerably higher than LH during all phases of the estrous cycle in cattle (Schallenberger et al., 1984; Walters and Schallenberger, 1984; Walters et al., 1984). Interpulse frequencies for FSH range from approximately 25 minutes during the preovulatory phase to 30-50 minutes during the mid-luteal phase of the estrous cycle (Walters and Schallenberger, 1984; Walters et al., 1984) with pulse amplitudes declining during the preovulatory period. Thus,

P_4 appears to exert only slight reductions in FSH pulse frequency in cattle. Evidence suggests that E_2 exerts an inhibitory effect on FSH release via the pituitary just prior to the preovulatory gonadotropin surge (Kesner and Convey, 1982; Walters and Schallenberger, 1984). Additionally, exogenous E_2 administered to ovariectomized heifers reduced FSH concentrations to precastration levels (Kesner and Convey, 1982). Follicular production of inhibin may also regulate plasma FSH concentrations during periods of follicular growth (De Jong, 1979; De Paolo et al., 1979; Henderson and Franchimont, 1982; Padmanabhan et al., 1984), possibly through decreasing pituitary release of FSH.

Low basal concentrations of P_4 and peak follicular production of E_2 potentiate the onset of behavioral estrus and trigger the preovulatory surge of LH and FSH from the anterior pituitary (Schams et al., 1977). Although estrous behavior in cattle may be induced by estrogen treatment alone (Melampy et al., 1957), a period of P_4 pre-exposure enhances the sensitivity of brain centers responsible for estrous behavior to E_2 (Melampy et al., 1958). Therefore, P_4 priming, during the luteal phase of the cycle, may be an important aspect of estrous expression (see also: McEwen et al., 1982; Pfaff and McEwen, 1983). During physiological states in which P_4 priming is absent, as occurs in heifers approaching puberty (Gonzalez-Padilla et al., 1975; Schams et al., 1981) or in cows following postpartum anestrous

(Schams et al., 1978; Peters, 1984), behavioral estrus does not accompany the first gonadotropin surge and ovulation. Additionally, duration and magnitude of P_4 exposure may influence subsequent luteal function by modulating preovulatory follicle development directly as was suggested by the data of Snook et al. (1969) in heifers treated with bovine LH antisera and McLeod and Haresign (1984) in the seasonally anestrous ewe. Similarly, ovulations occurring without the benefit of P_4 priming in pubertal and postpartum cattle often result in short-lived, less functional CL (Gonzalez-Padilla et al., 1975; Schams et al., 1978; 1981; Peters, 1984).

Elevated P_4 concentrations completely eliminate E_2 induction of estrous behavior and the gonadotropin surge (Hobson and Hansel, 1972; Short et al., 1979; Roche and Ireland, 1981; Ireland and Roche, 1982; Padmanabhan et al., 1982). With demise of the CL during the preovulatory period, plasma P_4 concentrations fall to below 1 ng/ml thus removing the negative feedback on GnRH and gonadotropin release rates. Follicular production of E_2 provides increasingly higher plasma E_2 concentrations, the magnitude of which is proportional to the size of the preovulatory gonadotropin surge (Walters and Schallenberger, 1984). Approximately 1 hour prior to the preovulatory surge of gonadotropins, Walters and Schallenberger (1984) observed a consistent decline in E_2 concentrations and suggested that

this event removed E_2 negative feedback on the hypothalamus and increased GnRH pulse amplitude which triggered the gonadotropin surge from the maximally responsive pituitary (Convey, 1973; Zolman et al., 1973; Kesner et al., 1981; Kesner and Convey, 1982). The surge of LH and FSH occur concomitantly near the onset of an 18 to 20 hour behavioral estrus (Trimberger and Hansel, 1955; Schams et al., 1977) and lasts 8 to 10 hours (Rahe et al., 1980; Kesner et al., 1981; Kesner and Convey, 1982; Walters and Schallenberger, 1984). The gonadotropin surge is terminated due to the refractoriness of the pituitary to GnRH (Kesner et al., 1981; Kesner and Convey, 1982).

During and immediately following the preovulatory gonadotropin surge, follicular (granulosa cell) production of E_2 and circulating levels of E_2 decline rapidly as follicular luteinization occurs (Chenault et al., 1975; Ireland and Roche, 1982). Plasma concentrations of E_2, P_4 and LH remain low throughout a majority of the metestrous phase. Pulses of LH are absent for 6 to 12 hours post-gonadotropin surge. Similarly, FSH pulse amplitudes are low; however, pulse frequencies remain at a rate comparable to that observed during the preovulatory gonadotropin surge (Walters and Schallenberger, 1984). Prior to ovulation and 4 to 12 hours following the gonadotropin surge, FSH concentrations become elevated as a result of an increase in FSH pulse amplitude (Kazmer et al., 1981; Hansel and Convey,

1983; Walters and Schallenberger, 1984). The second rise in FSH is of lower magnitude than the FSH surge and may be a result of reduced follicular inhibin production during the ovulatory process (De Jong, 1979; De Paolo et al., 1979; Henderson and Franchimont, 1981; Padmanabhan et al., 1984). The function of this secondary rise in FSH is currently unknown, however, FSH may play a role in oocyte maturation (Plachot and Mandelbaum, 1978) or in the recruitment of preantral follicles (Hansel and Convey, 1983).

Ovulation occurs on day 1 of the estrous cycle, approximately 24 to 30 hours after the preovulatory surge of LH and FSH (Chenault et al., 1975; Schams et al., 1977; Hansel and Convey, 1983). Following ovulation, development of CL function is reflected by the gradual increase in CL weight (Shemesh et al., 1976; Bartol et al., 1981) and P_4 content (Shemesh et al., 1976), plasma P_4 concentrations (Chenault et al., 1975; Peterson et al., 1976; Schams et al., 1977; Wise et al., 1981; Hansel and Convey, 1983) and P_4 production (Wise et al., 1981), and ovarian blood flow (Ford and Chenault, 1981; Wise et al., 1981). Follicular growth and atresia occur continuously throughout the estrous cycle (Rajakoski, 1960; Marion et al., 1968; Ireland and Roche, 1983) with moderate rises in plasma E_2 concentrations occurring responsively (Hansel et al., 1973; Hansel and Convey, 1983; Ireland and Roche, 1983).

During the early luteal phase, both LH and FSH secretion patterns may be characterized as high frequency-low amplitude (Rahe et al., 1980; Walters et al., 1984). The majority of LH and FSH pulses occur simultaneously during this period. Similarly, pulsatile episodes of E_2 and P_4 are high frequency. High amplitude pulses of E_2 follow LH pulses in 90 to 96% of the cases observed, while occurrence of low amplitude P_4 episodes were more similar to FSH pulsatile patterns (Walters et al., 1984). These gonadotropin-steroid pulsatile relationships become more obvious during the mid-luteal phase. By day 10 to 12 of the estrous cycle, frequency of LH episodes are significantly decreased (Rahe et al., 1980; Walters et al., 1984). Rahe and coworkers (1980) also described increased LH pulse amplitude during the mid-luteal phase, however, elevations in LH pulse amplitude were not evident in the data set of Walters et al. (1984). Pulse amplitude of FSH was similar during the early and mid-luteal phases, although FSH pulse frequency decreased during the mid-luteal phase. Nevertheless, 41% more FSH than LH episodes were observed during the mid-luteal phase. Low amplitude E_2 episodes were consistently preceded by pulses of LH and 92 to 100% of concurrent FSH and LH pulses and 97% of separate FSH pulses were followed by high amplitude episodes of P_4. In no case were separate FSH pulses associated with episodes of E_2 (Walters et al., 1984).

The dynamics of endocrine changes during the luteal phase support previously described roles for FSH and LH in follicular steroid production. Additionally, it would appear that FSH, in conjunction with LH, regulates P_4 secretion patterns. The major site of P_4 production is the CL (Shemesh et al., 1976). The bovine CL possesses both FSH (Mann and Niswender, 1983) and LH (Rao et al., 1979, 1983) receptors and responds to these gonadotropins, in vitro, with elevated P_4 production (Romanoff, 1966; Hixon and Hansel, 1979; Milvae et al., 1983).

In cyclic cattle, lifespan of the CL is regulated, to a large extent, by interactions between the ovary and uterus. During late diestrus-early proestrus phases of the cycle, estrogens produced by the developing, large antral follicle(s) are thought to initiate the process of luteal regression. Exogenous estrogens are known to shorten the lifespan of CL in cattle (Greenstein et al., 1958; Wiltbank, 1966; Eley et al., 1979). Luteolytic activity of estrogen is thought to be mediated through stimulation of uterine prostaglandin (PG)-$F_{2\alpha}$ synthesis and release (Thatcher et al., 1984b). The luteolytic activity of exogenous $PGF_{2\alpha}$ in cattle has been documented by several groups (Hansel et al., 1973; Hafs et al., 1974; Lauderdale, 1974; Thatcher and Chenault, 1976). Furthermore, episodic pulses of uterine venous $PGF_{2\alpha}$ (Nancarrow et al., 1973), and peripheral measurements of its primary metabolite,

15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM; Granstrom and Kindahl, 1982), are always associated with spontaneous luteal regression in cattle (Peterson et al., 1975; Kindahl et al., 1976; Betteridge et al., 1984). Uterine $PGF_{2\alpha}$ production requires a period of P_4-priming which increases the tissue's potential to synthesize $PGF_{2\alpha}$ while suppressing copious secretion of the luteolysin (Hansel et al., 1973; Horton and Poyser, 1976; Rothchild, 1981).

An indepth discussion of CL characteristics, function and mechanisms of regression will be reserved for a subsequent section of this chapter.

The Corpus Luteum Life Cycle

Origin and Development of Corpus Luteum Cells

Cells which make up the corpus luteum (CL) are derived from the preovulatory follicle. Two distinct luteal cell types have been described in CL of cattle (Donaldson and Hansel, 1965b; Ursely and Leymarie, 1979a,b; Koos and Hansel, 1981; Weber et al., 1984; Alila and Hansel, 1984), sheep (O'Shea et al., 1979, 1980; Fitz et al., 1982; Rogers et al., 1983, 1984), swine (Lemon and Loir, 1977; Lemon and Mauleon, 1982), rat (Kenny and Robinson, 1982), rabbit (Yuh et al., 1982) and human (Gaukroger et al., 1979). These cell types are described generally as small and large luteal cells. Current dogma suggests that small luteal cells are

derived from the theca interna and large luteal cells from the granulosa of the preovulatory follicle.

In the ewe, large luteal cells of the mature CL are approximately six-times larger than small luteal cells and occupy 25% or more of the CL volume (Niswender et al., 1976; Rodgers et al., 1984) as compared to approximately 18% by small luteal cells (Rodgers et al., 1984). Small luteal cells outnumber large luteal cells by nearly five to one. By far, the most numerous cell type within the mature ovine CL is endothelium (Rodgers et al., 1984), which attests to the high vascular density of this tissue.

Cellular mitotic activity in the CL is thought generally to be at a minimum (Donaldson and Hansel, 1965; Rodgers et al., 1984). Histological examination and DNA determinations of the bovine preovulatory follicle (6 hours following the onset of estrus) and CL throughout the estrous cycle (Donaldson and Hansel, 1965b) indicated that granulosa-luteal cell mitotic activity was reduced following the preovulatory surge of LH and ceased altogether by approximately day 4. The theca-derived luteal cells, however, mitosed freely until approximately day 7. Neovascularization continues in CL until a maximum metabolic potential is reached around days 10 to 12. In vivo exposure of developing bovine CL to hCG or LH (Donaldson et al., 1965a) stimulated mitotic activity in small luteal cells, vascular endothelium and stromal cells, whereas mitotic

figures were rarely observed in large luteal cells (Donaldson and Hansel, 1965). Thus, during CL development, LH appears to limit granulosa cell division and enhance theca-luteal cell mitosis (Donaldson and Hansel, 1965; Friedrich et al., 1975; McNatty and Sawers, 1975).

In addition, estimates of granulosa cell numbers in mature preovulatory follicles compare favorably with numbers of granulosa-luteal cells in the CL (McNatty, 1979; McNatty et al., 1984a; Rogers et al., 1984), suggesting that large luteal cells are derived solely from follicular granulosa cells. Furthermore, increases in follicular size are correlated positively with follicular cell numbers (McNatty, 1979; McNatty et al., 1982; Ireland and Roche, 1983). Thus, it may be assumed logically that events which regulate viability, proliferation and biosynthetic potential of preovulatory follicular cells markedly influence the functionality of the CL subsequently formed post-ovulation (see: McNatty, 1979; Richards, 1979, 1980; di Zerega and Hodgen, 1981; Murdoch et al., 1983).

Observations in the cow (Donaldson and Hansel, 1965; Alila and Hansel, 1984) and ewe (Fitz et al., 1981) support the hypothesis that CL growth occurs initially via hypertrophy and hyperplasia by both theca- and granulosa-derived luteal cells. However, these authors suggest that as the CL matures, theca-derived lutein cells enlarge to form large luteal cells. It has further been suggested that

the large luteal cells (granulosa- or theca-derived), which possess receptors for $PGF_{2\alpha}$, play a key role in CL regression (Fitz et al., 1982; Alila and Hansel, 1984; Hoyer et al., 1984).

Factors Regulating CL Steroidogenesis

The formation of luteal tissue is begun prior to ovulation as the preovulatory rise and surge release of LH promote several biochemical (Savard, 1973) and cytological (Donaldson and Hansel, 1965b; Blanchette, 1966a,b; Priedkalns and Weber, 1968; Enders, 1973; Van Blerkom and Motta, 1978; 1979) changes in both theca and granulosa cells of the preovulatory follicle.

Unlike the follicle, cells of the bovine CL lack the endoplasmic reticulum-associated 17-hydroxylase and 19-hydroxylase-aromatase enzyme systems (Savard and Telegdy, 1965; Savard, 1973). The obvious physiological manifestations of these biochemical alterations are the sudden loss of androgen and estrogen biosynthetic capacity by luteal cells (Savard and Telegdy, 1965; Ireland and Roche, 1982; Walters and Schallenberger, 1984). Thus, bovine CL exhibit a very abbreviated steroidogenic pathway from which P_4 is the primary end-product, with lesser amounts of 20β-hydroxy-4-pregnen-3-one (20β-P_4), pregnenolone (P_5), and 5α-reduced progestins (5α-P) being generated (Wise and Fields, 1978; Albert et al., 1982). Corpus luteum development and steroidogenic capacity may be

grossly assessed by monitoring plasma P_4 concentrations. Bovine CL mass increases rapidly from day 3 ($\leq$ 1 gram) to day 7 (4 gram) and is maintained at a weight of 5 to 6 grams from days 10 through 17. Likewise, plasma concentrations of P_4 are low ($\leq$ 1 ng/ml) on day 3, but rise rapidly to levels of approximately 5 ng/ml by day 7 and are maintained between 6 and 10 ng/ml from days 10 through 17 (Donaldson and Hansel, 1965b; Erb et al., 1971; Rao et al., 1979; Milvae and Hansel, 1983a). During regression of the CL, a loss in function (reduced plasma P_4 concentrations) consistently precedes any decline in CL weight.

Luteal steroidogenic potential is regulated by several factors. Luteinizing hormone is considered the primary luteotropin in cattle. During the past 20 years, a tremendous number of studies have been performed which support this concept. Administration of exogenous LH (Donaldson and Hansel, 1965a), hCG (Wiltbank et al., 1961), and GnRH-agonists, to induce elevated endogenous LH concentrations (Milvae et al., 1984), increased plasma P_4 concentrations and prolonged CL function in cattle. Several in vitro studies demonstrated increased P_4 production by dispersed luteal cells or luteal slices when incubated with LH or hCG (Hansel and Seifart, 1967; Hansel et al., 1973; Hixson and Hansel, 1979; Milvae and Hansel, 1983a; Tan and Biggs, 1984; Milvae et al., 1985). Antiserum to LH prevented this luteotropic effect of LH on luteal slices in

vitro (Hansel and Seifart, 1967) and induced a decline in CL weight and P_4 content of CL in intact heifers (Snook et al., 1969). In hysterectomized heifers, LH treatment increased P_4 content of CL (Brunner et al., 1969) while hysterectomy plus hypophysectomy or hypophysectomy alone caused a decline in P_4 content of CL, lower plasma P_4 concentrations, and a reduction in CL weight in cattle (Henricks et al., 1969). Lastly, oxytocin and $PGF_{2\alpha}$ induced CL regression was circumvented by administration of LH or GnRH to cyclic cattle (Hansel and Seifart, 1967; Thatcher and Chenault, 1976). Therefore, maximal function of the bovine CL is contingent upon LH stimulation.

Direct binding studies, using radiolabelled ligands, revealed the presence of specific, high affinity-low capacity receptors for LH/hCG in bovine luteal cell membranes (Rao et al., 1979, 1983) and intracellular organelles (Rao et al., 1983). Furthermore, luteal concentrations of LH receptor sites are correlated with P_4 secretion during the estrous cycle. Other ligands (to be discussed later) which bind specifically to luteal tissue include FSH (Mann and Niswender, 1983), $PGF_{2\alpha}$ (Kimball and Lauderdale, 1975; Rao et al.,1979; Bartol et al., 1981; Fitz et al., 1982), PGE_1 and PGE_2 (Kimball and Lauderdale, 1975; Fitz et al., 1982), and E_2 (Glass et al., 1984).

Recent application of cell separation techniques have enabled researchers to better characterize and evaluate the

function of specific luteal cell populations with regard to their roles in P_4 production and luteolysis. As described previously, small luteal cell numbers exceed large luteal cells by approximately five-fold although large luteal cells comprise more of the CL volume. In cattle (Ursely and Leymarie, 1979a,b; Koos and Hansel, 1981; Weber et al., 1984), sheep (Fitz et al., 1982; Rodgers and O'Shea, 1982; Rodgers et al., 1983), and swine (Lemon and Loir, 1977; Lemon and Mauleon, 1982) incubations enriched in large luteal cells produce consistently more P_4 than equivalent numbers of small luteal cells. However, only small luteal cells were capable of responding to LH/hCG stimulation, as determined by a significant increase in P_4 production over nonstimulated cells. This observation suggested, as was demonstrated by Fitz and coworkers (1982), that the luteotropic effect of LH/hCG was due to the interaction of LH/hCG with specific receptor sites on small luteal cells. Small cell steroidogenesis was also stimulated by cyclic AMP and PGE_2. The deficiency of LH/hCG receptors in the large luteal cell population and a constant secretion of P_4 in the presence or absence of secretagogues (Fitz et al., 1982) indicate that peak steroidogenic activity in large luteal cells does not require receptor-mediated cyclic AMP generation as it does in small luteal cells (Hoyer et al., 1984). Instead, maximum potential for P_4 production by

large lutein cells appears to be dependent upon precursor (primarily cholesterol) availability.

Lemon and Mauleon (1982) demonstrated an interaction between small and large luteal cell types in the porcine CL with regard to P_4 production. These authors utilized a superfusion system in which medium was pumped from one chamber, containing small luteal cells, through a second chamber, containing large luteal cells, and vise-versa. Progesterone production by the small cell to large cell 'in series' superfusion exceeded (20%) the sum total of P_4 produced by each cell type alone. No such increase in P_4 production was detected when the superfusion was conducted with cell populations in a reversed order. Thus, products derived from small luteal cells stimulated P_4 production by large luteal cells. When steroid precursors were added to superfusions of either small or large luteal cells, P_5 was metabolized to P_4 equally well by both cell types. In contrast, only large luteal cells responded to exogenous cholesterol with increased P_4 production compared to control cells superfused without cholesterol. Metabolism of cholesterol by large cells was not stimulated by exogenous LH. Pulses of exogenous LH to small luteal cell superfusions stimulated P_4 production responsively. However, P_4 production in response to LH was not affected by the addition of cholesterol to small luteal cells. Lemon and Mauleon (1982) suggested that local transfer of

cholesterol from small to large luteal cells was responsible for enhanced P_4 production by large luteal cells. Furthermore, LH-stimulated P_4 production and cholesterol mobilization in small luteal cells would provide additional substrate to large luteal cells. The end result would be a coordinated increase in P_4 production by both cell types.

The following discussion will summarize some of the important processes involved in cellular cholesterol regulation. Pertinent references may be found in reviews by Savard, 1973; Brown et al., 1981; Kaplan, 1981; Savion et al., 1982; Strauss et al., 1982.

Steroidogenic cells, and extrahepatic cells in general, synthesize very little cholesterol. Instead, the bulk of cholesterol required for steroidogenesis and membrane synthesis is derived from plasma and stored as cholesteryl esters within the cell.

Approximately 70% of the total plasma cholesterol pool resides within low density lipoprotein (LDL) particles in an esterified form. These LDL particles are approximately 220 Å in diameter and contain a core of nearly pure cholesteryl ester surrounded by two copies of the 250,000 Dalton glycoprotein, apoprotein B. Cellular uptake of cholesterol involves binding of LDL to specific, high affinity receptors which become localized on specialized regions of the plasma membrane called coated pits (Pastan and Willingham, 1981a,b). Receptor recognition of specific regions of

apoprotein B ensures the selective uptake of LDL. Receptor binding affinity to LDL is Ca^{+2}-dependent, the importance of which will become obvious. Receptor-LDL complexes are rapidly internalized enmass via receptor mediated endocytosis (Pastan and Willingham, 1981b). Coated pits, containing numerous receptor-LDL complexes, invaginate and pinch off to form endocytotic coated vesicles which ultimately fuse with lysosomal membranes in the cytoplasm. Receptor-ligand complexes dissociate allowing the LDL receptors to be recycled for further use. Dissociation is thought to occur as a result of low Ca^{+2} concentrations within the endocytotic vesicle and lysosome. Hydrolysis of LDL within the lysosome frees cholesterol for use or storage within the cell.

Intracellular cholesterol concentrations are highly regulated by the cell. As intracellular cholesterol concentrations rise following hydrolysis of LDL in the lysosomes, cellular cholesterol biosynthesis is suppressed via a decrease in the activity of a pivitol enzyme in the cholesterol biosynthetic pathway, HMG-CoA reductase. Secondly, acyl-CoA:cholesterol acyltransferase activity is enhanced, thus catalyzing the re-esterification of cholesterol for storage. Most importantly, LDL receptor biosynthesis is suppressed, preventing further uptake of cholesteryl ester-rich LDL from the plasma.

These data suggest that small and large luteal cells also adjust to intracellular cholesterol concentrations in accordance with changing cellular requirements. Thus, large lutein cells, which synthesize approximately 80% of the total P_4 in unstimulated CL (Hoyer et al., 1984), would be expected to maintain a higher cholesterol requirement than small luteal cells. Furthermore, it may be suggested that large luteal cells in the pig (Lemon and Mauleon, 1982) were in a cholesterol-deficient state, with regard to their maximum steroidogenic capacity, since P_4 production was elevated following exposure to exogenous cholesterol. Conversely, small luteal cells, which utilize cholesterol less rapidly, may possess larger stores of intracellular cholesterol and would not be expected to exhibit elevations in P_4 production in response to exogenous cholesterol (Lemon and Mauleon, 1982). Demand for cholesterol by LH-sensitive small luteal cells increases following stimulation with LH or cyclic AMP. However, intracellular stores are apparently sufficient to accomodate this demand (Lemon and Mauleon, 1982).

Luteinizing hormone and cyclic AMP increase cholesterol esterase and cholesterol side chain cleavage (SCC) enzyme activities, lipoprotein uptake and P_4 production (Savard, 1973; Bisgaier et al., 1979; Caffrey et al., 1979a; Strauss et al., 1982). However, specific mechanisms of LH action on luteal cells are not defined clearly.

Intracellular Ca^{+2} and cyclic AMP concentrations, and cyclic AMP-dependent protein kinase activation are known to play essential roles in transduction of receptor-mediated signals (see: Rasmussen, 1981). General concepts regarding the mechanism of action of peptide hormones on target cells have centered around intracellular accumulation of Ca^{+2} and cyclic AMP, and activation of cyclic AMP-dependent protein kinase(s) (Catt and Dufau, 1976; Marsh, 1976). This enzyme mediates the effects of cyclic AMP via phosphorylation of specific substrates which in turn regulate cell function. Cyclic AMP-dependent protein kinase(s) has been localized in the cytoplasm and numerous cellular oganelles (plasma membrane, mitochondria, ribosomes, nucleus and endoplasmic reticulum) suggesting that diversity of cellular responses to cyclic AMP may depend on availability of specific substrates within compartmentalized regions of the cell (Langan, 1973).

Recent evaluation of membrane structure and function has expanded our understanding of mechanisms involved in receptor-mediated information flow into the cell. Over the past decade, it has become apparent that the organization of phospholipids and proteins within membranes is more complex than described by the "fluid mosaic" membrane model of Singer and Nicolson (1972). Several components of biological membranes (i.e., receptors, enzymes, phospholipids) are asymmetrically distributed between the

inner and outer bilayer (Rothman and Lenard, 1977; Lodish and Rothman, 1979). Furthermore, membrane components may aggregate preferentially in specific domains of the bilayer structure (Chapman et al., 1979; Karnovsky et al., 1982). Assembly of phospholipid and protein domains facilitate events such as intercellular communications (Loewenstein, 1970; Garfield et al., 1979; Hertzberg et al., 1981), endocytosis (Pearse, 1980; Schlessinger, 1980; Pastan and Willingham, 1981a,b), ion movement and enzymatic activity (Savard, 1973; Strauss et al., 1982; Lentz et al., 1983). Membrane components are in a state of constant reorganization, synthesis and breakdown in response to external stimuli. Current data suggest that, in addition to Ca^{+2} and cyclic AMP messenger systems, rapid and transient alterations in membrane phospholipid composition, biosynthesis and metabolism are essential in stimulus-response coupling in cells (Hirata and Axelrod, 1980; Davis et al., 1981; Crews, 1982; Farese, 1983; Milvae et al., 1983). Phospholipid methylation (Hirata and Axelrod, 1980; Crews, 1982; Milvae et al., 1983) and phosphotidylinositol (PI) metabolism (Davis et al., 1981; Crews, 1982; Farese, 1983; Nishizuka, 1984b) are two mechanisms by which membrane phospholipids are transiently altered in response to receptor-ligand binding.

Methylation of phospholipids is dependent upon two phospholipid methyltransferase (PMT) enzymes which are

distributed on the inner (PMT-1) and outer (PMT-2) aspects of the cell membrane bilayer. Likewise, the phospholipid substrates for PMT-1 (phosphatidylethanolamine, PE) and PMT-2 (phosphatidyl-N-monomethylethanolamine, PME) are asymmetrically distributed within the membrane. Formation of PME requires the PMT-1 catalyzed transfer of a single methyl group to PE. Conversion of PME to PC requires two successive methyl transfers by PMT-2. Successive methylations of PE by this system result in translocation of methylated phospholipids (PME and phosphatidylcholine, PC) toward the outer aspects of the membrane bilayer.

As a result of these chemical alterations in phospholipid composition, the membrane becomes less viscous. Enhanced membrane fluidity is thought to result from the accumulation of deeply embedded PME within the phospholipid bilayer. With the increase in membrane fluidity, lateral mobility of membrane proteins is potentiated thereby enhancing the coupling of surface receptors with adenylate cyclase and cyclic AMP formation (Rimon et al., 1978; Axelrod, 1983). Additionally, formation and deposition of PC in the outer phospholipid layer of the membrane promotes the unmasking of cryptic membrane receptors. Thus, receptor availability is enhanced (Hirata and Axelrod, 1980; Crews, 1982).

Milvae et al. (1983) demonstrated the importance of phospholipid methylation in expression of LH-stimulated P_4

production by dispersed bovine luteal cells. Incorporation of [^{3}H]-methyl-groups into PME, PC and PI was stimulated by LH, in vitro. An endogenous methyl-donor, S-adenosyl-L-methionine (SAM), enhanced LH-induced P_4 production while methylation inhibitors, 3-deazaadenosine (DZA) and S-adenosyl-L-homocysteine (SAH), prevented the luteotropic action of LH on dispersed luteal cells. Cyclic AMP effects on P_4 production were not influenced by DZA or SAH, supporting the view that inhibition of phospholipid methylation prevents coupling of ligand-receptor complexes to adenylate cyclase, cyclase activation and cyclic AMP generation in the bovine CL.

The degree of membrane phospholipid methylation also appears to influence cellular Ca^{+2} fluxes, possibly via regulation of calcium ion channels and Ca^{+2}-dependent adenosinetriphosphatase (ATPase) activity (Hirata and Axelrod, 1980; Crews, 1982). Regulation of cell function by calcium ion concentrations and intracellular Ca^{+2} binding proteins, such as calmodulin, have been reviewed by several authors (Cheung, 1979; Means, 1981; Rasmussen, 1981). Receptor mediated Ca^{+2} influx occurs secondarily to methylation of phospholipids and, like cyclic AMP generation, is prevented by methylation inhibitors. Activation of Ca^{+2}-dependent phospholipase A_2 parallels the influx of Ca^{+2}, as demonstrated by elevated free arachidonic acid and lysolecithin (lysophosphatidylcholine)

concentrations following receptor activation (Crews, 1982). These observations suggest that hydrolysis of newly synthesized methylated phospholipids by Ca^{+2}-dependent phospholipases serve to regulate methylated phospholipid-induced membrane effects, viz., increased fluidity, Ca^{+2} influx, receptor availability. It is interesting to note that Ca^{+2} is not required for membrane phospholipid methylation or cyclic AMP generation in several cell systems, although many of the physiological responses to cyclic AMP are Ca^{+2}-dependent (Rasmussen, 1970).

In addition to phospholipid methylation, considerable interest has been directed toward receptor-mediated alterations of the phosphatidate-inositide cycle (Crews, 1982; Farese, 1983; Nishizuka, 1984b). There are two known mechanisms by which the phosphatidate-inositide cycle may be affected by receptor activation. The first involves the hydrolysis of phosphatidylinositides to diacylglyceride (DG) and inositol phosphates via phospholipase C catalyzed removal of the phosphorylated inositide head group. This mechanism is thought to regulate plasma membrane Ca^{+2} fluxes and intracellular Ca^{+2} distribution. The phosphatidylinositides are localized primarily in the inner phospholipid layer of the plasma membrane bilayer, and membranes of endoplasmic reticulum and mitochondria as mono-, di- and triphosphorylated inositol phospholipids (PI, PIP and PIP_2, respectively). These phospholipids bind Ca^{+2} avidly and

provide a releasable Ca^{+2} pool upon hydrolysis. Recent evidence, reviewed by Nishizuka (1984b), suggests that PIP_2, rather than PI or PIP, is rapidly degraded following receptor activation. Once hydrolysis of PIP_2 is initiated in the plasma membrane the process is thought to self-propagate as a result of elevated free Ca^{+2} concentrations, increased Ca^{+2}-dependent phospholipase activity, and extended hydrolysis of phosphatidylinositides within the plasma membrane and intracellular organelles. One metabolite of PIP_2 hydrolysis, inositol triphosphate, may serve as a mediator for Ca^{+2} release from intracellular stores (Nishizuka, 1984b). A second metabolite, DG, arises following phospholipase C catalyzed hydrolysis of PIP_2, PIP or PI. Diacylglycerol may serve as a precursor for the regeneration of phosphatidylinositides as well as PE and PC. The phosphatidylinositides and DG contain primarily arachidonate at fatty acid position -2. Thus, hydrolysis by Ca^{+2}-dependent phospholipase A_2 provides free arachidonic acid for use in prostaglandin and hydroxyperoxylipid biosynthetic pathways (Ramwell et al., 1977), and various lysolecithins, many of which are required for membrane fusion reactions during uptake and secretion of products (Crews, 1982). Additionally, DG activates the Ca^{+2}- and phospholipid-dependent protein kinase C (Nishizuka, 1984a,b). Protein kinase C activation has been implicated in the elicitation of cell proliferation (Nishizuka, 1984a)

and inhibition of gonadal steroidogenesis (Welsh et al., 1984). It is interesting to note that protein kinase C serves as a receptor for tumor promoting phorbol esters (Nishizuka, 1984a,b) which are thought to irreversibly activate the enzyme. Phorbol esters possess a similar chemical structure to DG, the endogenous activator of protein kinase C. Phorbol esters have been shown to inhibit gonadotropin-induced steroidogenesis, in vitro (Welsh et al., 1984), supporting a similar role for endogenous DG in the regulation of steroid production. Therefore, steroidogenesis may be antagonized as a consequence of phosphatidylinositide hydrolysis.

Conversely, de novo phosphatidate-inositide biosynthesis may be important in the mediation of gonadotropin effects on steroidogenesis (Davis et al., 1981; Crews, 1982; Farese, 1984). Several studies have demonstrated that LH and ACTH stimulate biosynthesis of phosphatidylinositides and phosphatidic acid (PA) in gonadal and adrenal cortex cells, respectively (Davis et al., 1981; Strauss et al., 1982; Farese, 1984). Data reviewed by Farese (1984) indicated that identical responses were triggered by cyclic AMP, suggesting that phospholipid biosynthesis occurred after cyclic AMP generation. Both hormone- and cyclic AMP-induced phosphatidate-inositide biosynthesis, and steroidogenesis are blocked by protein synthesis inhibitors, puromycin and cycloheximide.

Apparently, the two enzymes responsible for PA biosynthesis, viz., glycerol-3-phosphate acyltransferase and diglyceride kinase, are substrate activated and require protein synthesis. Hormone activation of these enzyme systems is indirect and thought to occur by increasing glycerol phosphate, glucose, fatty acid and diglyceride availability through stimulation of glycogenolysis, glycolysis, glucose uptake and lipolysis. Phosphatidylinositol kinase catalyzes the conversion of PA plus ATP to PI, and subsequent phosphorylations to PIP and PIP_2.

Phosphatidate-inositol biosynthesis parallels steroidogenic activation in adrenal and luteal tissues following exposure to ACTH and LH, respectively. Furthermore, addition of certain polyphosphorylated phospholipids, viz., the phosphatidylinositides and cardiolipin, to adrenal cortex and luteal cell cultures stimulated steroid biosynthesis in a dose dependent manner (Strauss et al., 1982; Farese, 1984). Effects of these "steroidogenic" phospholipids are not prevented by cycloheximide or puromycin.

The site of phospholipid action in both adrenal cortex and luteal cells appears to be the mitochondria. Specifically, steroidogenic phospholipids stimulate cholesterol side chain cleavage (SCC) by intact cells and acetone powders of luteal mitochondria (Strauss et al., 1982;

Farese, 1984), although the mechanism through which this is accomplished remains unknown.

At present, it is known that trophic hormones, such as LH and ACTH, stimulate steroidogenesis by influencing several biochemical steps. Free intracellular cholesterol concentrations are elevated via enhanced cellular uptake of cholesterol from plasma and mobilization of cholesterol from intracellular stores following cholesterol esterase activation. Apparently, hormone induced elevations in free intracellular cholesterol are not affected by cycloheximide, but are blocked by cytochalasin B suggesting that microfilaments, but not protein synthetic events, are crucial in the acquisition of substrate for steroid biosynthesis (Gemmel and Stacy, 1977; Sawyer et al., 1979; Silavin et al., 1980; Rasmussen, 1981; Gwynne and Condon, 1982). Microtubule involvement in steroidogenesis is, however, less clear with evidence reported both pro (Gemmel and Stacy, 1977; Sawyer et al., 1979) and con (Gwynne and Condon, 1982).

In addition to elevating intracellular substrate concentrations, LH and ACTH increase mitochondrial content of cholesterol, presumably as a result of enhanced cholesterol transfer from cytosol to mitochondria (see: Rasmussen, 1981, for references). This event requires protein synthesis and microfilament action. Hormone- and cyclic AMP-induced biosynthesis of the "steroidogenic" phospholipids are also dependent on protein synthetic

events, although their stimulatory effects on mitochondrial SCC activity are not (Strauss et al., 1982; Farese, 1984). Furthermore, mitochondrial SCC activity is substrate- and Ca^{+2}-dependent. Thus, it appears as though hormone-induced steroidogenic activity involves primarily processes which increase intracellular cholesterol content and transfer of cholesterol from the cytosol to the SCC enzyme complex, viz., sterol carrier proteins (see: Savard, 1973, for references). Translocation of cholesterol across the mitochondrial membranes may be regulated by the phosphatidylinositides and other "steroidogenic" phospholipids (Strauss et al., 1982; Farese, 1984), the synthesis of which is dependent on some, as yet undetermined, protein synthetic event. Increased SCC activity may also involve structural changes in the enzyme-phospholipid domain (Savard, 1973; Strauss et al., 1982), and altered mitochondrial Ca^{+2} concentrations (Crews, 1982; Farese, 1984).

It is becoming more apparent that membrane phospholipid dynamics, in addition to cyclic AMP and Ca^{+2} second messenger effects, are of paramount importance in cellular information flow and stimulus-response coupling. The reader is referred to Rasmussen (1981) for further discussions on this broad and rapidly developing area of cell physiology.

Following side chain cleavage of cholesterol, newly synthesized P_5 is transferred out of the mitochondria to the endoplasmic reticulum. Here, the membrane bound

3β-hydroxy-Δ^5-steroid dehydrogenase/Δ^5-Δ^4 isomerase enzyme complex (HSD) catalyzes the rapid conversion of P_5 to P_4, the primary steroid end product of the CL. These latter steps in the biosynthesis of P_4 do not appear to be regulated by trophic hormones. However, luteal P_4 production may be modified by local and systemic mechanisms involving steroids, arachidonic acid metabolites, and ovarian peptide hormones.

Intraovarian control of CL function

The dependency of CL P_4 production on LH has been extensively documented in the literature, as has the importance of uterine-derived $PGF_{2\alpha}$ in luteolytic events. However, the level and duration of luteal P_4 production may also be determined by the local ovarian environment in which the CL resides.

A large body of evidence supports a role for steroids in the regulation of steroidogenic enzyme activities in the ovary, testes, adrenal and placenta (see: Rothchild, 1981; Gower and Cooke, 1983). Caffrey et al. (1979b) demonstrated that the affinity of HSD for its product, P_4, was substantially greater than for P_5 substrate and that P_4 (10-50 μg) inhibited HSD activity in ovine CL preparations. Thus, suggesting that P_4 may modify its own production via substrate inhibition of HSD activity in the CL. Testosterone and E_2 (10 μg) were also found to be potent inhibitors of HSD activity, in vitro (Caffrey et al., 1979b;

Gower and Cooke, 1983), but not in vivo (Caffrey et al., 1979b). Similarly, microgram concentrations of testosterone and E_2 were found to inhibit LH-stimulated P_4 production but not cyclic AMP accummulation in dispersed luteal cells collected from 2 to 6 months of gestation in cattle. (Williams and Marsh, 1978). In vivo luteolytic interactions were reported between E_2 and $PGF_{2\alpha}$ in hysterectomized ewes (Gengenbach et al., 1977), and between E_2 (0.5 μg) and oxytocin (200 mIU) in dispersed luteal cells collected from cyclic cattle (Tan and Biggs, 1984). However, Hixon and Hansel (1979) were unable to demonstrate similar luteolytic effects with subnanogram doses of E_2 or E_2 plus $PGF_{2\alpha}$ in dispersed bovine luteal cells from days 12 and 13 of the cycle. Further support for a direct E_2-CL interaction was provided in a recent study (Glass et al., 1984) in which E_2 receptors were characterized in large and small luteal cells throughout the estrous cycle of the ewe. Concentrations of E_2 receptor rose gradually during the estrous cycle and were localized primarily in large lutein cells. Additionally, Sairam and Berman (1979) provided evidence that synthetic estrogens prevented normal coupling of the gonadotropin-receptor complex to its catalytic subunit, thus preventing the initiation of intracellular gonadotropic-induced responses.

Discrepancies with regard to the inhibitory role(s) of E_2 on CL P_4 biosynthesis may be due to differences in dosage

of steroid employed, specie and status (cyclic or pregnant) of the animal from which luteal tissue was collected, and in vitro versus in vivo experimental conditions. Although doses of E_2 which inhibit CL P_4 biosynthesis seem high and nonphysiological, they do compare favorably with concentrations reported in follicular fluid of preovulatory follicles (see: Fortune and Hansel, 1979). Therefore, the CL may be exposed to high intraovarian concentrations of various steroids, viz., testosterone and E_2, as would be expected during preovulatory follicle development in the late diestrum. The potential inhibitory nature on luteal P_4 production exhibited by high concentrations of these steroids supports a functional role in the initiation of CL regression. In this regard, it is noteworthy that P_4 plasma concentrations begin to fall prior to any detectable rise in peripheral plasma E_2 concentrations or initiation of uterine $PGF_{2\alpha}$ pulsatile activity (Chenault et al., 1975; Peterson et al., 1975).

Beyond the luteotrophic effects initiated by LH, P_4 production may be "protected" from steroid negative feedback effects by several intraovarian processes.

For example, binding of steroids to proteins for short-term storage, viz., steroid carrier proteins (Goddard et al., 1980; Willcox, 1983) or long-term storage in cytoplasmic granules (Gemmell et al., 1974; Gemmell and Stacy, 1979; Sawyer et al., 1979) may reduce intracellular

concentrations of free steroid and reduce feedback inhibition, in vivo. Furthermore, steroid sulfation or conjugation to fatty acids, viz., lipoidal steroids (Hochberg et al., 1979; Albert et al., 1980; Schatz and Hochberg, 1981; Albert et al., 1982), within the CL would accomplish a similar function. Lastly, ovarian blood flow parallels luteal P_4 production in cattle (Ford and Chenault, 1981; Wise et al., 1982) thus providing an additional means of reducing CL and ovarian steroid concentrations through elevated clearance rates.

In recent years, several ovarian peptides, including steroid-binding proteins (Willcox, 1983), LH-binding inhibitors (Kumari et al., 1982; Ward et al., 1982), gonadotropin releasing hormone (GnRH)-like peptides (Ying et al., 1981; Fraser, 1982; Sharpe, 1982), angiogenic factor (Gospodarowicz and Thakral, 1978), oxytocin (Fields et al., 1983; Rodgers et al., 1983; Wathes et al., 1983), vasopressin (Wathes et al., 1983) and relaxin-like peptides (Fields et al., 1982) have been discovered. Although biochemical evidence suggests that these peptides are produced by the CL and/or follicle, little information is available concerning their physiological roles in ovarian processes.

Of the peptides mentioned above, oxytocin has been evaluated most thoroughly. It is known that CL of the cow and ewe contain high concentrations (μg/g) of oxytocin

(Fields et al., 1983; Flint and Sheldrick, 1983; Flint et al., 1983; Wathes et al., 1983) which is synthesized and stored in the large lutein cells (Rodgers et al., 1983). The bovine CL contains neurophysin, lending further support for the local synthesis of oxytocin within the CL (Wathes et al., 1983). Additionally, circulating oxytocin concentrations are rapidly reduced following ovariectomy (Schams et al., 1982) and concentrations of immunoreactive oxytocin were greater in ovarian venous plasma versus uterine arterial or jugular venous plasma (Flint and Sheldrick, 1982; Walters et al., 1984). Concentrations of oxytocin and neurophysin in luteal tissue (Wathes et al., 1984) and plasma (Sheldrick and Flint, 1981; Flint and Sheldrick, 1983; Schams, 1983; Walters et al., 1984) parallel CL P_4 production during the luteal phase, although the decline in oxytocin concentrations occur prior to P_4 during CL regression. Oxytocin and P_4 are secreted from the CL in a pulsatile fashion. Walters et al. (1984) reported that only 29% of P_4 episodes were associated with pulses of oxytocin during the early luteal phase, due largely to the occurrence of fewer oxytocin than P_4 pulses at this time. During the mid-luteal phase 86% of P_4 and oxytocin episodes occurred simultaneously. Remarkably, 97 to 100% of oxytocin pulses, during all periods of the luteal phase, were associated with P_4 pulses. These observations suggest that

similar mechanisms may regulate the secretion of P_4 and oxytocin from the CL.

Several lines of evidence support a systemic role for oxytocin in the process of CL regression (reviewed in a later section). Additional data suggests that oxytocin may have an action within the ovary as well. Evidence reviewed by Wathes (1984) indicates that oxytocin (and vasopressin) may be involved in P_4 production during CL development since oxytocin (and vasopressin) can promote P_4 biosynthesis in testicular cells through the inhibition of LH-stimulated androgen biosynthesis. Oxytocin probably acts by selective suppression of 17α-hydroxylase and C17-20 desmolase activities. Follicular androgen and estrogen biosynthesis is suppressed in favor of P_4 biosynthesis prior to and following the preovulatory LH surge. Furthermore, oxytocin has been measured in preovulatory follicles, ovarian tissue (including all follicles) devoid of CL, and newly formed CL (Wathes, 1984; Wathes et al., 1984). Therefore, preovulatory follicle and luteal oxytocin biosynthesis, possibly induced by LH, may prevent C-19 and C-18 steroid biosynthesis and promote P_4 formation in CL. High levels of oxytocin in CL during the mid-luteal phase may reduce follicular E_2 production in a similar manner (Wathes, 1984). Results of Tan et al. (1982) also demonstrated stimulatory effects of low levels of oxytocin (2 to 20 mIU) in CL collected from pregnant cattle. Likewise, exogenous

oxytocin, administered to cyclic cattle on days 12 and 13, increased P_4 content of CL on day 14 (Mares and Casida, 1963). Conversely, high doses of oxytocin (200 to 400 mIU) were inhibitory to in vitro P_4 biosynthesis by CL of cyclic (Tan and Biggs, 1984) and pregnant (Tan et al., 1982) cattle.

Luteolytic prostaglandins appear to mediate oxytocin secretion from the CL (Flint and Sheldrick, 1982, 1983; Schallenberger et al., 1984). Similarly, $PGF_{2\alpha}$ or analogues of $PGF_{2\alpha}$ stimulate rapid degranulation of large lutein cells (Heath et al., 1983) which are known to be the synthetic and storage site of oxytocin in the CL (Rodgers et al., 1983). Consistent with the hypothesis of oxytocin storage in granules are observations which demonstrate parallel patterns of luteal oxytocin content (Wathes et al., 1984) and secretion (Sheldrick and Flint, 1981; Flint and Sheldrick, 1983; Schams, 1983; Walters et al., 1984) with large luteal cell granule content (Gemmell et al., 1974). Based on the nearly absolute synchrony with which tonic oxytocin and P_4 pulses occur, in vivo (Walters et al., 1984), and the ability of $PGF_{2\alpha}$ to induce oxytocin secretion, it may be suggested that luteal prostaglandins are involved in tonic oxytocin and P_4 secretion during the luteal phase of the estrous cycle. Evidence of luteal prostaglandin biosynthesis (Shemesh and Hansel, 1975a;

Lukaszewska and Hansel, 1979; Milvae and Hansel, 1985a) strengthens this supposition.

Bovine luteal tissue contains massive quantities (2 to 3 mg/g) of arachidonic acid, the bulk of which is esterified to phospholipids (Lukaszewska and Hansel, 1979, 1980). Thus, the potential for arachidonic acid metabolism in either the cyclooxygenase or lipoxygenase pathway is great. Metabolic pathways utilizing arachidonic acid as precursor have been reviewed previously (Ramwell et al., 1977). Characterization of local biosynthetic patterns and functions of luteal prostaglandins and other arachidonic acid metabolites have provided additional insight into mechanisms controlling luteal P_4 production and regression.

Shemesh and Hansel (1975b) demonstrated an acute increase in ovarian venous $PGF_{2\alpha}$ concentrations following injection of arachidonic acid into CL on days 12 or 13 in cyclic cattle. Peripheral plasma P_4 concentrations fell rapidly from approximately 6 ng/ml to 2.5 ng/ml, and remained constant until day 15 or 16 when the experiment concluded. Additionally, CL weights on day 15 or 16 were less in arachidonic acid-treated (4.3 ± 1.4 g) than vehicle-treated (6.3 ± 0.5 g) cattle. Several studies have demonstrated an inverse relationship between luteal $PGF_{2\alpha}$ and P_4 content or production (Patek and Watson, 1976; Lukaszewska and Hansel, 1979; Rothchild, 1981). However, the means by which elevated luteal $PGF_{2\alpha}$ contents reduce P_4

production remain to be elucidated. Exogenous $PGF_{2\alpha}$ administration to cattle (Lukaszewska and Hansel, 1979), sheep (Diekman et al., 1978) and rats (Hichens et al., 1974; Grinwich et al., 1976) initiates a reduction in P_4 production and the loss of LH receptors or luteal sensitivity to LH. The authors suggested that loss of LH receptor numbers or reduced sensitivity to LH were the consequence rather than cause of luteolysis since reduced P_4 production occurred considerably earlier than either of the LH related phenomenon.

The $PGF_{2\alpha}$-induced decline in luteal P_4 production described above may be related more closely to changes in ovarian vascular resistance. Blood flow to the CL-bearing ovary parallels closely luteal P_4 production (Niswender et al., 1975; Ford and Chenault, 1981) and $PGF_{2\alpha}$ is known to reduce ovarian blood flow and luteal P_4 production (Nett et al., 1976; Niswender et al., 1976). Therefore, $PGF_{2\alpha}$ effects may be mediated through increasing ovarian vascular resistance. However, metabolite requirements are generally thought to dictate the degree of blood flow to a tissue. In this light, reduced luteal metabolic activity may initiate the accummulation of $PGF_{2\alpha}$ and subsequent decline in luteal blood flow.

Progesterone biosynthetic activity of bovine CL collected during the estrous cycle is highly correlated (r^2=.93) with luteal prostacyclin (PGI_2) biosynthesis

(Milvae and Hansel, 1983a). Sun et al. (1977) demonstrated that PGI_2 was the predominant prostaglandin product following bovine CL membrane metabolism of PGH_2. Furthermore, PGI_2 stimulated luteal P_4 biosynthesis in vitro and in vivo (Milvae and Hansel, 1980b). A recent report by Milvae and Hansel (1985) demonstrated that indomethacin inhibition of prostaglandin formation early in the estrous cycle shortened luteal lifespan in cattle. Indomethacin was administered into the uterine lumen twice daily from days 4 through 6. In conjunction with previous reports, these authors proposed that luteotropic products of the cyclooxygenase pathway, viz., PGI_2 and PGE_2, may be required for normal CL development. The origin of these prostaglandins may include both the CL and uterus. Additionally, synthesis and content of PGI_2 and $PGF_{2\alpha}$ in bovine CL are greatest early in the cycle (Milvae and Hansel, 1983a). Luteal prostacyclin content was approximately four times greater than $PGF_{2\alpha}$ early in the cycle although ratios of PGI_2 and $PGF_{2\alpha}$ gradually decline in CL age increased (Milvae and Hansel, 1983a). Milvae et al. (1985) provided several lines of evidence suggesting that lipoxygenase products, viz., 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and 5-hydroxyeicosatetraenoic acid (5-HETE), regulate P_4 production by the selective inhibition of prostacyclin synthetase activity and PGI_2 production in bovine CL. Large quantities of 5-HETE are present in bovine

CL on days 10, 15 and 18 of the cycle and 5-HETE inhibited in vitro PGI_2 and P_4 biosynthesis in a dose dependent fashion without influencing $PGF_{2\alpha}$ production. Simultaneous addition of LH was unable to prevent the 5-HETE-induced reduction in luteal cell PGI_2 and P_4 production, in vitro. In related experiments (Milvae et al., 1985), arachidonic acid metabolism through the lipoxygenase pathway was inhibited by nordihydro-guaiaretic acid (NDGA). When NDGA was added to dispersed luteal cells, PGI_2 production was increased after a 2 hour incubation. Progesterone and $PGF_{2\alpha}$ synthesis had increased slightly, although not significantly ($P>.05$). Arachidonic acid plus NDGA increased luteal production of both PGI_2 and $PGF_{2\alpha}$ but not P_4, suggesting an antagonism between PGI_2 and $PGF_{2\alpha}$ in the regulation of luteal P_4 synthesis (Milvae et al., 1985). This possibility is also suggested by the declining ratio of PGI_2 and $PGF_{2\alpha}$ contents in bovine CL as luteolysis approaches (Milvae and Hansel, 1983a). Collectively, these data suggest that HETE, and perhaps other lipoxygenase products, are involved in luteolysis by blocking synthesis of the luteotropin, PGI_2, and allowing additional arachidonic acid to be shunted toward pathways involved in synthesis of luteolytic prostaglandins.

Similar mechanisms controlling biosynthesis of luteotropic and luteolytic prostaglandins may also occur

within the uterus (Milvae et al., 1985; Milvae and Hansel, 1985).

Uterine Control of CL Function

There is little doubt that the uterus plays a key role in regulation of CL lifespan during the estrous cycle. Loeb (1923) first demonstrated that extirpation of the guinea pig uterus resulted in extended CL function. Subsequent reports in cattle (Wiltbank and Casida, 1956; Anderson et al., 1961; Malven and Hansel, 1964; Anderson et al., 1965), sheep (Wiltbank and Casida, 1956; Anderson et al., 1969), pigs (Spies et al., 1958; Du Mesnil Du Buisson, 1966) and horses (Ginther and First, 1971) provided similar results following surgical removal of the uterus. Thus, a basis for uterine involvement in the luteolytic process was developed. The nature of this utero-ovarian relationship was further defined following observations that cattle (Bland, 1970) and sheep (McCrackin and Caldwell, 1960) with congenital absence of the uterine horn (uterus unicornis) adjacent to the CL bearing ovary exhibited prolonged luteal lifespans and cycle lengths which suggested local regulation of CL function by the ipsilateral uterine horn. Additionally, normal luteal regression occurred following hemihysterectomy of the contralateral, but not the ipsilateral uterine horn in

cyclic cattle and sheep (Inskeep and Butcher, 1966; Moor and Rowson, 1966; Ginther, 1974, 1981).

As a consequence of these data, Wollmerhaus (1964) and Ginther (review, 1974) examined the anatomy and histology of the utero-ovarian vascular architecture as a possible route of exchange between the uterine horns and their adjacent ovaries. In cattle and sheep, species which exhibit local regulation of CL function by the uterus, exchange of luteolytic substances from the uterine venous effluent (ovarian vein, International Committee of Veterinary Anatomical Nomenclature, 1968) into the ovarian arterial supply is thought to occur just below the ovarian vascular pedicle. At this point, the ovarian artery follows an extremely tortuous and convoluted path over the surface of the ovarian vein (containing both uterine and ovarian venous blood) thus maximizing the area of contact between the two vessels. Histologically, the adventitial layers of each vessel were substantially thinner in the regions of vein-artery apposition (Wollmerhaus, 1964; Del Campo and Ginther, 1974). From an anatomical and histological perspective, these observations favored the existence of a functional venoarterial exchange system between uterine horns and their adjacent ovaries.

A series of physiological experiments, utilizing surgical anastomoses of ovarian veins or ovarian arteries in hemihysterectomized cattle and sheep, provided conclusive

support for the functional ovarian vein and artery components of the local uteroovarian pathway (Ginther, 1974; 1981). In such experiments, luteal regression occurred when either ovarian vein or ovarian artery blood from the contralateral, uterine-intact side was diverted to the appropriate vessel on the ipsilateral, hemihysterectomized side. Conversely, CL were maintained if the surgical anastomosis became occluded. Thus, it became clear that the uterus was producing a blood borne product which could move from the uterine venous drainage into the ovarian arterial supply and initiate the process of luteolysis in a local manner.

As early as 1940, Hechter and coworkers suggested that some uterine-derived product may be responsible for regulation of CL lifespan. However, it was not until the resurgence of prostaglandin research in the 1950's that the identity of the uterine luteolytic factor was elucidated. The chemical structures of several prostaglandins, including $PGF_{2\alpha}$, were confirmed in 1962 (Nelson et al., 1982) and by 1969, Pharris and Wyngarden provided evidence that the vasoactive prostaglandin, $PGF_{2\alpha}$, may be the uterine luteolytic substance in the rat. Subsequently, the luteolytic activity of exogenous $PGF_{2\alpha}$ in cattle has been documented extensively (Hansel et al., 1973; Hafs et al., 1974; Lauderdale, 1974; Thatcher and Chenault, 1976). Several studies have provided evidence indicating that $PGF_{2\alpha}$

is an endogenous luteolysin in cattle. For example, elevated levels of $PGF_{2\alpha}$ in uterine venous drainage (Nancarrow et al., 1973; Shemesh and Hansel, 1975), uterine tissue (Shemesh and Hansel, 1975) and uterine flushings (Lamothe et al., 1977; Bartol et al., 1981a) coincide closely with the period of expected luteal regression. In addition, luteolysis is prevented in cows immunized passively against $PGF_{2\alpha}$ (Fairclough et al., 1981) or following intrauterine administration of the prostaglandin synthetase inhibitor, indomethacin, late in the estrous cycle (Lewis and Warner, 1977).

The biological half-life of $PGF_{2\alpha}$ is very short owing to its rapid metabolism by the lungs (Piper et al., 1970) and uterine endometrium (Thatcher et al., 1984; Chapter 3). Thus, measurements of its primary metabolite, 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM; Granstrom and Kindahl, 1982), provide an index of uterine $PGF_{2\alpha}$ production. Peripheral concentrations of PGFM are significantly correlated with the uterine production of $PGF_{2\alpha}$ (Thatcher et al., 1984; Chapter 3) and concentrations of $PGF_{2\alpha}$ in uterine flushings (Bartol et al., 1981a). Furthermore, three to five pulsatile episodes of PGFM were always temporally associated with the decline in plasma P_4 concentrations during luteolysis in cattle (Peterson et al., 1975; Kindahl et al., 1976; Betteridge et al., 1984). Collectively, these data indicate

that the endogenous uterine luteolysis in cattle is likely to be $PGF_{2\alpha}$.

Finally, in keeping with the concept of a local venoarterial pathway, transfusion of $PGF_{2\alpha}$ from the uterine venous drainage into the adjacent ovarian arterial supply was demonstrated. In an early study, Goding et al. (1971) induced luteal regression, as demonstrated by the precipitous decline in plasma P_4 concentrations, following the infusion of physiological concentrations of $PGF_{2\alpha}$ into the uterine venous drainage of diestrous cattle. Hixon and Hansel (1974) reported elevated $PGF_{2\alpha}$ concentrations in the uterine venous drainage (ovarian vein) and ovarian artery, ipsilateral to the CL bearing ovary, as a result of the intrauterine deposition of $PGF_{2\alpha}$. Similarly, Thatcher et al. (1984b) verified the functionality of the countercurrent exchange system ($PGF_{2\alpha}$) during the period of normal luteal regression in cyclic cattle.

Regulation of Uterine $PGF_{2\alpha}$ Production

Demise of the cyclic CL is uterine and $PGF_{2\alpha}$-dependent, as reviewed above. However, interplay between ovarian estrogen, progesterone and oxytocin with the uterine endometrium regulate the timing and magnitude of luteolysin production during the estrous cycle. Estrogens, of follicular origin, appear to constitute an important stimulatory component regulating uterine $PGF_{2\alpha}$ production and CL regression. Destruction of ovarian follicles in

cattle (Villa-Godoy et al., 1981) and sheep (Karsch et al., 1970; Ginther, 1971) result in prolonged luteal lifespans and heavier CL compared to follicle-intact controls. Furthermore, administration of estrogens to late-luteal phase cows and ewes resulted in production of $PGF_{2\alpha}$ by the uterus (Barcikowski et al., 1974; Thatcher et al., 1984b; Chapter 3) and premature CL regression (Greenstein et al., 1958; Wiltbank, 1966; Stormshak et al., 1960; Caldwell et al., 1972; Eley et al., 1979). As anticipated, luteolytic doses of E_2 were ineffective in hysterectomized (Brunner et al., 1969; Akbar et al., 1971; Caldwell et al., 1972) and indomethacin-treated (Warren et al., 1979; Barcikowski et al., 1974) cattle and sheep. In another study (Loy et al., 1960), E_2 administration to cattle on day 1 or 4 of the estrous cycle had no effect on CL examined on day 14. However, treatment with P_4 caused a reduction in CL weight, P_4 content, and number of functional luteal cells. Administration of exogenous P_4 for 1 to 5 days post-estrus in cattle and sheep results consistently in shorter interestrous intervals (Woody et al., 1967; Ginther, 1968, 1969; Thwaites, 1971; Lawson and Cahill, 1983), presumably as a result of premature development and activation of the uterine $PGF_{2\alpha}$ secretory system (Baird et al., 1976; Ottobre et al., 1980). These data suggest that the uterus requires a period of P_4 exposure in order to develop the capacity to secrete luteolytic levels of $PGF_{2\alpha}$ in response to

estrogens. For example, in the cow (Skjerven, 1956; Martinov and Lovell, 1968) and ewe (Brinsfield and Hawk, 1973) the degree of endometrial lipid droplet accumulation varies cyclically, being more prevalent during P_4 than E_2 dominated phases of the estrous cycle. Additionally, exogenous P_4 stimulated accumulation of endometrial lipid droplets while E_2 administration decreased lipid stores (Brinsfield and Hawk, 1973). Finally, Hansel et al. (1975) extracted large quantities of arachidonic acid, the principle substrate for $PGF_{2\alpha}$ synthesis, from bovine endometrium between days 10 and 14 of the luteal phase. Collectively, these results support the concept that P_4 directs endometrial accumulation of lipid and prostaglandin precursor concentrations during the estrous cycle.

Estrogens induce the luteolytic response during late diestrus.

Timing and magnitude of the uterine luteolytic response are dependent on circulating patterns and concentrations of P_4 and E_2 which, in turn, regulate the degree of uterine sensitivity to ovarian steroids, viz., endometrial P_4 and E_2 receptor populations. Estrogen is known to stimulate synthesis of endometrial estrogen and P_4 cytoplasmic receptors (R_c) (Clark et al., 1977; Koligian and Stormshak, 1977a). Conversely, P_4 inhibits synthesis of E-R_c and reduces nuclear retention of the estrogen-receptor complex in endometrial tissue (Clark et al., 1977; Koligian and

Stormshak, 1977a; Katzenellenbogan et al., 1980). Progesterone also reduces endometrial synthesis of its own receptor (Schrader and O'Malley, 1978; Walters and Clark, 1980). With this in mind, direct measurements of steroid receptors in bovine and ovine endometrium revealed elevated concentrations of $E\text{-}R_c$ during proestrus and metestrus, and low $E\text{-}R_c$ concentrations during diestrus (Senior, 1975; Koligian and Stormshak, 1977b; Henricks and Harris, 1978). Additionally, Zelinski et al. (1982) demonstrated a decline in bovine endometrial $P_4\text{-}R_c$ concentrations from the proestrus to diestrus phases of the estrous cycle. Thus, as the CL develops and plasma E_2:P_4 ratios decline, concentrations of endometrial $E\text{-}R_c$ fall precipitously. Plasma P_4 thereby inhibits the uterus from responding in an estrogen-directed fashion. However, elevated plasma P_4 concentrations progressively suppress endometrial production of its own receptor such that estrogen-induced events (i.e., prostaglandin biosynthesis) are gradually removed from P_4 inhibition.

Induction of uterine $PGF_{2\alpha}$ production by E_2 may be attributed partially to E_2-dependent increases in activities of cyclooxygenase (Huslig et al., 1979) and phospholipase A_2 (Dey et al., 1982). Calcium is probably involved in activating phospholipase A_2 since this enzyme is Ca^{+2} dependent (Brockerhoff and Jensen, 1974). Estrogen was also

shown to increase uterine Ca^{+2} availability in swine (Geisert et al., 1982).

Estrogen-induced luteolysis is prevented by inhibition of uterine DNA dependent RNA synthesis with actinomycin D (French and Casida, 1973) suggesting that the process of $PGF_{2\alpha}$ production requires protein synthesis. Furthermore, Roberts et al. (1976) and McCracken et al. (1981, 1984) provided evidence for estrogen induction of endometrial oxytocin receptors following a period of P_4 priming in sheep. Exogenous oxytocin stimulates uterine $PGF_{2\alpha}$ production in cyclic cattle (Newcomb et al., 1977; Milvae and Hansel, 1980) and sheep (Roberts et al., 1975, 1976; McCracken, 1981, 1984) and causes premature CL regression in uterine-intact (Armstrong and Hansel, 1959; Hansel and Wagner, 1960; Milne, 1963), but not hysterectomized (Ginther et al., 1967) or indomethacin-treated (Cooke and Homeida, 1983) females. In addition, luteal function was extended in ewes immunized against oxytocin (Sheldrick et al., 1980; Schams et al., 1983). Based on these results and reports concerning secretion of oxytocin and P_4 by the CL (reviewed earlier), McCracken and coworkers (1981, 1984) proposed a working hypothesis for CL regression in cyclic ewes. Briefly, their proposed sequence of events are: 1) declining endometrial P_4 receptor population reduces P_4 dominance on the uterus during late diestrus and permits follicular E_2 to stimulate endometrial estrogen and oxytocin

receptor formation and activate enzymes essential for $PGF_{2\alpha}$ biosynthesis; 2) circulating luteal (or pituitary) oxytocin binds to newly synthesized endometrial receptors and initiates $PGF_{2\alpha}$ secretion; 3) plasma P_4 concentrations decline slowly, further reducing its influence on the uterus; 4) uterine-derived $PGF_{2\alpha}$ induces a rapid dumping of luteal oxytocin which reinforces uterine $PGF_{2\alpha}$ production and 5) $PGF_{2\alpha}$ initiates a rapid decline in plasma P_4 concentrations associated with CL demise.

Available data indicate that $PGF_{2\alpha}$ is the primary uterine luteolysin in numerous species (Horton and Poyser, 1976). However, recent data implicate additional arachidonic acid metabolites in the luteolytic process. For example, the 13,14-dihydro metabolite of $PGF_{2\alpha}$ was as effective as equivalent amounts of $PGF_{2\alpha}$ in causing CL regression in heifers (Milvae and Hansel, 1983). Arachidonic acid metabolites of the lipoxygenase pathway may also be physiological regulators of luteal lifespan (Milvae et al., 1985). Lipoxygenase products, such as 5-hydroxy-eicosatetraenoic acid (5-HETE), specifically inhibit luteal synthesis of prostacyclin, a potent luteotropin (Milvae and Hansel, 1980, 1983), thereby reducing luteal P_4 production, in vitro. When in vitro synthesis of 5-HETE and other lipoxygenase products was blocked by nordihydroguaiaretic acid NDGA, luteal cell prostacyclin and P_4 production were increased without influencing $PGF_{2\alpha}$ production. Further,

twice daily intrauterine administration of NDGA to cyclic heifers on days 14 to 18 extended luteal function approximately 5 days beyond control heifers. These data suggest that lipoxygenase products, of uterine and/or ovarian origin, are involved in the luteolytic process. In a final study, Milvae and Hansel (1985) provided evidence that uterine and/or ovarian prostaglandins are required for normal CL development and function in cattle. Intrauterian administration of indomethacin to cyclic heifers on days 4, 5 and 6 caused decreased P_4 production and early CL regression. The authors implied that luteotropic prostaglandins, such as PGE_2 and prostacyclin, may be involved in luteal development.

Conceptus-Associated Events During Early Pregnancy

An opportunity for establishment of pregnancy is provided with each estrous cycle. As a consequence of luteal P_4 production, glandular epithelium of the uterine endometrium develops morphological/ultrastructural characteristics, and functions of an active secretory tissue (Skjerven, 1956b; Marinov and Lovell, 1968; Wathes and Wooding, 1980). Several studies have demonstrated increased endometrial tissue content or uterine secretion of amino acids, proteins, fatty acids, lipids, carbohydrates, and certain ions during the luteal phase of the estrous cycle or

in response to exogenous P_4 (cow: Skjerven, 1956a; Fahning et al., 1967; Carlson et al., 1970; Roberts and Parker, 1974; Hansel et al., 1975; Bartol et al., 1981; sheep: Brinsfield and Hawk, 1973; Roberts and Parker, 1976; pig: Murray et al., 1972; Knight et al., 1974; Bazer, 1975; Bazer et al., 1977; human: Shapiro et al., 1980). In addition, elevated P_4 concentrations reduce uterine tone and myometrial contractility (Hays and Van Demark, 1953; Lehrer and Schindler, 1974). Thus, during the first 15 to 16 days post-estrus, a complex embryotrophic environment is established within the uterine lumen.

During early pregnancy there is a clear requirement for developmental synchrony between the conceptus and endometrium. Embryo transfer studies in cattle and sheep indicate that a small degree of asynchrony (± 1 or 2 days) is tolerated with respect to stage of transferred conceptus and recipient uterine development (Rowson and Moor, 1966; Rowson et al., 1969; Sreeman, 1978; Seidel, 1981). However, pregnancy success is highest when synchronization is precise (Cook and Hunter, 1978).

As described earlier, P_4 administered early in the estrous cycle is thought to initiate premature development and activation of the uterine $PGF_{2\alpha}$ secretory system (Baird et al., 1976; Ottobre et al., 1980). This system was utilized recently to evaluate the role of P_4 pretreatment, early in the estrous cycle, on the embryonic state of the

ovine uterus (Lawson and Cahill, 1983). Progesterone administered during the first 4 days of the ovine estrous cycle accelerated development of the uterine luminal environment such that the day 6 P_4-treated uterus provided acceptable support for normal development of transferred 10 day old embryos. Luteolysis had not occurred in 8 of 12 ewes slaughtered on day 25. No day 10 conceptuses survived following transfer to control (no P_4 pretreatment) uteri on day 6. Estrous cycles in these ewes were of normal length. These data suggest that uterine development and complexity of its luminal environment progress sequentially under the influence of P_4. Further, the conceptus must develop in synchrony with this environment to survive.

The inability of embryos to develop beyond the blastocyst stage, when cultured in vitro (Wright and Bondioli, 1981) or confined within the oviduct (Wintenberger-Torres, 1956; Murray et al., 1971; Pope and Day, 1972; Heap et al., 1979), illustrate that unique components of the uterine environment become essential for further conceptus development. The essentiality of this relationship may be due, in part, to increased complexity of conceptus nutrient and hormonal requirements following blastulation (Daniel, 1971; Biggers and Borland, 1976). Additionally, Lawson et al. (1983) demonstrated an active interaction between the uterine environment and rate of conceptus development in sheep. In two experiments,

sixteen-cell embryos (day 4) were transferred to uteri of ewes on days 4, 6 or 7 of the estrous cycle. The embryos were then recovered and evaluated 4 or 6 days later. Embryos transferred to older, more advanced uteri were viable when recovered (days 8 to 12 of the estrous cycle) and exhibited accelerated growth rates during the 4 to 6 days in utero as compared to synchronously transferred controls. A third experiment examined conceptuses on days 12, 14 or 15 of the estrous cycle following synchronous versus asynchronous embryo transfers, as described above. Results indicated that viability and accelerated growth rates of asynchronously transferred embryos were halted by day 11 or 12 post-estrus. Further, these conceptuses were unable to maintain CL function in recipient ewes. Similarly, P_4 has been implicated in regulating ovine conceptus growth during normal pregnancy (Bindon, 1971) and in superovulated recipients of embryo transfers (Wintenberger-Torres, 1968; Wintenberger-Torres and Rombauts, 1968). Presumably, P_4 effects on embryonic growth are mediated by alterations in the uterine luminal milieu. Collectively, these data demonstrate that the conceptus is capable of responding to physiochemical cues in its uterine environment or conversely, the uterus exerts a regulatory influence on some aspects of conceptus development during early pregnancy. Either of these viewpoints may explain how small degrees of developmental asynchrony between the uterus

and conceptus are tolerated in large domestic species (Rowson and Moor, 1966; Rowson et al., 1969; Sreenan, 1978; Seidel, 1981). However, there appears to be a developmental limit, beyond which the conceptus may not "catch up" to its uterine environment and prevent luteolysis (Lawson et al., 1983).

Regardless of this physiological flexibility, a considerable percentage of embryos do not survive beyond day 30 of pregnancy in large domestic species. In cattle, the majority of embryonic mortality occurs between days 16 and 26 of pregnancy (Hawk et al., 1955; Boyd et al., 1969; Ayalon, 1978; Ball, 1978; Hawk, 1979). Dramatic changes in bovine conceptus growth, differentiation (Melton et al., 1951; Chang, 1952; Greenstein and Foley, 1958a, b; Greenstein et al., 1958; King et al., 1982) and endocrine activity (Shemesh et al., 1979; Chenault, 1980; Lewis et al., 1982; Eley et al., 1983; Bartol et al., 1985) occur during this relatively short period. Pregnancy success between days 16 to 30 of gestation depends upon 1) appropriate physiochemical communication between conceptus and endometrium such that CL maintenance is achieved, and 2) the initiation of placental (cotyledon and caruncle) development and function. "Maternal recognition of pregnancy" (Short, 1969), occurring by days 16 to 17 in cattle (Betteridge et al., 1980; Northey and French, 1980; Dalla Porta and Humblot, 1983), and day 12 in sheep (Moor

and Rowson, 1966; Rowson and Moor, 1967) and swine (Dhindsa and Dziuk, 1968), represents the first period during early gestation when production of conceptus signals becomes essential for luteal maintenance and continued endometrial secretory support. The conceptus does not appear to be essential for CL maintenance or initiation of an embryonic uterine environment prior to maternal recognition of pregnancy. This was clearly demonstrated by Betteridge et al. (1980) when pregnancies were established following synchronous embryo transfers as late as day 16 in cyclic cattle. Additionally, noticable histological alterations in endometrial development between pregnant and cyclic cattle do not occur prior to day 17 (King et al., 1981, 1982). Thus, pregnancy associated events occurring prior to maternal recognition of pregnancy may be thought of as being mediated primarily by the CL and endometrium, whereas events following this period are under direction of the conceptus.

Conceptus Development (Days 15 to 30)

As noted earlier, developmental phases including maternal recognition of pregnancy through definitive placentome formation occur between days 16 and 30 of gestation in cattle. The large degree of embryonic mortality during this period attests to the critical nature of these developmental processes with regard to establishment and maintenance of pregnancy.

On day 16 of gestation, the bovine conceptus is characteristically bilaminar (trophectoderm and endoderm), with more advanced conceptuses exhibiting varying degrees of mesodermal germ layer development (Chang, 1952; Greenstein and Foley, 1958 a, b). Trophectodermal and endodermal germ layers initiate a phase of rapid elongation by days 15 to 16 of pregnancy. Lengths of extraembryonic membranes vary tremendously during this period (1.5 to 225 mm; Hawk et al., 1955; Greenstein and Foley, 1958b; Betteridge et al., 1980) with mean diameters calculated between 50 and 90 mm.

Histological and ultrastructural evaluation of rapid and progressive transition of porcine conceptus forms, viz. spherical, tubular and elongated filamentous, between days 10 and 12 of gestation (Geisert et al., 1982) provided evidence that rapid conceptus elongation occurs as a result of trophectodermal and endodermal reorganization and not hyperplasia. Whether or not this phenomenon occurs during conceptus elongation in the cow or sheep is not known.

By day 17, mean conceptus extraembryonic membrane length reaches approximately 150 mm (Chang, 1952; Greenstein and Foley, 1958b). Histochemical evaluation of trophectodermal cell types (days 16 to 33; Greenstein et al., 1958) based on staining characteristics with Sudan Black B or Oil Red O (demonstration of lipids), Periodic-Acid-Schiff (PAS; demonstration of glycogen, glycoproteins), and phloxine-methylene blue (demonstration of cytoplasmic

basophilia or acidophilia) delineated three distinct types of cells by day 16 and 17. These included: 1) PAS (+), trophoblastic giant cells (GC) which were occasionally binucleated at this stage; 2) undifferentiated trophoblast "stem cells"; and 3) normal columnar trophoblast cells which consistently contained basally located lipid inclusions. The embryo proper, viz. germinal or embryonic disc, is notably oval in shape by day 17 and demonstrates signs of initiating organogenesis, as indicated by the appearance of the primitive groove and node. Additionally, ultrastructural appearance of day 17 endometrial epithelium was indistinguishable in pregnant versus cyclic cattle (King et al., 1981), suggesting that no physical interaction between conceptus and endometrium had occurred at this stage of pregnancy.

Conceptus adhesion to maternal endometrium was noted to occur by days 18 to 20 (Wathes and Wooding, 1980; King et al., 1980, 1981), although no microvillous interdigitation was observed. This physical interaction between the conceptus and endometrium is consistent with the observed reduction in uterine epithelial cell height by day 18 of pregnancy (Wathes and Wooding, 1980; King et al., 1981) and the apparent migration of binucleated trophoblastic GC into the uterine epithelial cell layers (Wathes and Wooding, 1980). Extraembryonic membranes continue to elongate, extending throughout the gravid and nongravid uterine horns

by days 19 and 20. In addition, numbers of binucleated GC continue to increase, constituting as much as 20% of the total trophectodermal cell population on day 20 (Wathes and Wooding, 1980).

Between days 18 and 20, amnionic formation is completed and the allantoic diverticulum develops via an evagination of embryonic hindgut splanchnopleure (Greenstein and Foley, 1958a; Greenstein et al., 1958). Conversely, the highly vascularized yolk sac reaches its developmental peak by day 20 and begins regressing thereafter as the allantois emerges (Greenstein et al., 1958). This period of development marks a coordinated transition from conceptus-derived (choriovitelline placenta) to maternal-derived (chorioallantoic placenta) nutritional support.

Mutual microvillous interdigitation between trophoblastic and endometrial epithelium is initiated by day 24 (King et al., 1980, 1981; Wathes and Wooding, 1980) while definitive attachment occurs by day 27 (King et al., 1980). Additionally, immature fetal cotyledonary villi are apparent adjacent to maternal caruncles from approximately days 27 to 30 of gestation (Greenstein et al., 1958; King et al., 1981). Considerably more lipid accumulation was noted in trophectodermal epithelium from day 27 onward (King et al., 1980) suggesting that functional exchange of nutrients from maternal to conceptus tissues was accelerated following definitive attachment. There is a continued increase in the

number of bi- and multinucleated GC reported in trophectoderm and endometrial epithelial cell layers throughout this period (Greenstein et al., 1958; King et al., 1980; Wathes and Wooding, 1980). Multinucleated GC were reported to comprise 25% of total endometrial epithelial cell population and 50% of cell area in the gravid uterine horn on day 24 (Wathes and Wooding, 1980).

Regression of the yolk sac is complete by day 24. However, allantois expansion and vascularization are not completed until approximately days 30 to 33 (Greenstein et al., 1958). Diameter of the allantois ranges from 0.8 to 5.5 cm on day 24 to over 35 cm on day 33. Dramatic expansion and vascularization of the allantois is initiated between days 28 to 30 (Greenstein et al., 1958). Continued expansion of allantoic fluid volume and membrane diameter during the first two months of gestation forces extraembryonic membranes into apposition with the endometrium, thus facilitating development of firm cotyledon-caruncle attachment (Eley et al., 1978). Establishment of a vascularized chorioallantoic placenta preceeds and is essential for promotion of substantial fetal weight gains during later gestation (Eley et al., 1978).

Conceptus Signals and Maternal Recognition of Pregnancy

A functional CL is required if pregnancy in cattle is to persist. Maintenance of the CL during pregnancy depends on the presence of a viable conceptus within the uterine

lumen and its ability to appropriately "signal" a receptive maternal system, such that processes mediating cyclic regression of the CL are circumvented. In cattle, maternal recognition of pregnancy (Short, 1969) must be initiated by days 16 to 17 post-estrus if pregnancy is to become established. For example, Betteridge et al. (1980) demonstrated that synchronous embryo transfer to recipients on or prior to day 16, but not day 17 post-estrus, resulted in normal pregnancies. Additionally, nonsurgical embryo removal from pregnant cattle on days 16 through 19 resulted in extended CL maintenance and interestrous intervals (range 4 to 8 days) compared to nonmated controls (Northey and French, 1980; Dalla Porta and Humblot, 1983). Embryo removal prior to day 16 of pregnancy, viz. days 9, 13, 14, and 15, had no effect on cycle length or occurrence of luteolysis. These data provided clear evidence that CL maintenance required the presence of a viable conceptus within the uterine lumen of cattle by days 16 to 17 post-estrus. Further data indicated that twice daily intrauterine injections of homogenized day 17 or 18 conceptuses (Northey and French, 1980) or freeze-killed day 16 conceptuses (Dalla Porta and Humblot, 1980) on days 14 through 18 or days 15 through 19, respectively, also extended luteal function. Northey and French (1980) noted that intrauterine injections of conceptus homogenates did not stimulate luteal P_4 production, but extended the

interval to CL regression. Intrauterine administration of two freeze-killed, day 12 embryos/injection had no effect on CL lifespan (Dalla Porta and Humblot, 1983). Thus, potential for bovine conceptus production of potent, biologically active substances by day 16 of pregnancy was supported, as was their involvement in prolongation of CL function.

Subsequent studies in cattle suggested that there are probably several interdependent strategies by which conceptus signals may initiate maintenance of the CL during early pregnancy. Potential effectors, or biologically active signals, which are synthesized and secreted by bovine conceptuses (days 13 to 27) include steroids (Shemesh et al., 1979; Chenault, 1980; Gadsby et al., 1980; Eley et al., 1983; Chapter 2), prostaglandins (Shemesh et al., 1979; Lewis et al., 1982; Lewis, 1984; Lewis and Waterman, 1984), and proteins (Masters et al., 1982; Bartol et al., 1985; Chapter 4). This subject has been reviewed recently by Thatcher et al. (1984a, b; 1985) with respect to maternal recognition of pregnancy in cattle.

In several studies, higher plasma P_4 concentrations were detected in pregnant versus bred-nonpregnant or cyclic cattle from as early as days 9 to 10 post-estrus (Henricks et al., 1971; Ford et al., 1979; Lukaszewska and Hansel, 1980), suggesting a luteotropic role of the conceptus during early pregnancy. However, pregnancy-related elevations in

P_4 concentrations were not supported by others (Batson et al., 1972; Folman et al., 1973; Hasler et al., 1980). Progesterone production by dispersed luteal cells was stimulated by homogenates and aqueous extracts of day 18 bovine conceptuses (Beal et al., 1981). Progesterone stimulatory activity of conceptus extracts was lost following heating or removal by dialysis of components less than a relative molecular weight (Mr) of 12,000 Daltons. Similarly, the elongating ovine conceptus contains products which stimulate luteal P_4 production, in vitro (Godkin et al., 1978). The product(s) responsible for this luteotropic activity in ewes is not proteinaceous since 100 μg of conceptus protein had no effect on synthesis of P_4 or cyclic AMP by dispersed ovine luteal cells (Ellinwood et al., 1979). Furthermore, no LH-like activity was detected in bovine (day 16 to 20; Henricks and Poffenbarger, 1984) or ovine (day 14 to 15; Ellinwood et al., 1979) conceptuses as demonstrated under bioassay and RIA conditions, respectively. Thus the presumptive luteotropin(s) may be a steroid or prostaglandin. Marsh (1970) demonstrated that PGE_2 stimulated bovine luteal production of P_4 in vitro. However, evaluation of in vivo PGE_2 effects on bovine luteal function and maintenance are not clearly indicated. Prostaglandin-E_2 administered into the uterine lumen alone (Gimenez and Henricks, 1983; Chenault, 1983) or in combination with E_2 (Reynolds et al., 1983) extends luteal

function only slightly beyond cessation of intrauterine PGE_2 treatments. No stimulation of luteal P_4 production was noted in vivo. Systemic P_4 concentrations declined within 12 h following cessation of PGE_2 treatment (Gimenez and Henricks, 1983) or during the treatment period (Reynolds et al., 1983) suggesting that PGE_2 (plus E_2) will not prevent production and transfer of uterine luteolytic substances, but affects luteal function directly (Marsh, 1970; Henderson et al., 1977; Reynolds et al., 1981). Others have reported no effect of intrauterine PGE_2 administration on CL maintenance in cattle (Dalla Porta and Humblot, 1983; Chenault et al., 1984). As in cattle, reports in sheep indicate that luteal function may be prolonged with PGE_2 for only short periods beyond the time of normal luteolysis (Pratt et al., 1979; Huie et al., 1981; Magnuss et al., 1981). In sheep, numerous studies have demonstrated that the CL becomes refractory to exogenous $PGF_{2\alpha}$ during early pregnancy (Inskeep et al., 1975; Mapletoft et al., 1976b; Pratt et al., 1977; Silvia and Niswender, 1984) or following PGE_2 administration (Pratt et al., 1977; Henderson et al., 1977; Reynolds et al., 1981). Furthermore, PGE_2 is synthesized by bovine (Shemesh et al., 1979; Lewis et al., 1982) and ovine (Hyland et al., 1982; Lacroix and Kann, 1982) conceptuses and endometrium. Luteal protective substances, originating from the gravid uterine horn in cattle, were demonstrated by Del Campo et al. (1980). In

this study, uterine horns were isolated surgically and embryos transferred to uterine horns contralateral or ipsilateral to the CL containing ovary. In three or four cases, pregnancies in the ipsilateral horn maintained CL viability, whereas all pregnancies on the contralateral side failed to maintain CL function. However, when the vein draining the uterus of the contralateral, pregnant horn was anastomosed surgically with the ipsilateral, nonpregnant uterine venous drainage, luteal regression was prevented in three of three cows. Thus, it was apparent that some blood-borne product(s) of pregnancy was able to circumvent the luteolytic effects of the nonpregnant uterine horn. Similar findings were reported in the ewe (Mapletoft et al., 1976b). These results supported previous data demonstrating existence of a unilateral uteroovarian relationship (veno-arterial pathway) during early pregnancy (Del Campo et al., 1977) and luteolysis (Ginther, 1974; Ginther et al., 1974) in cattle. Collectively, data generated in cattle and sheep suggest that conceptus (and/or uterine)-derived luteal protective substances provide the CL with some resiliency against $PGF_{2\alpha}$-induced luteolysis during early pregnancy. Evidence supports PGE_2 as a possible luteal protective product of conception.

Movement of substances to and from the pregnant uterus and ipsilateral ovary may be regulated, to some degree, by blood flow rates and intraovarian vascular hemodynamics

(Lamond and Drost, 1974; Ford and Chenault, 1980). During early pregnancy (days 14 to 18) in cattle, blood flow to the gravid uterine horn is transiently increased (Ford et al., 1979; Ford and Chenault, 1980; Ford, 1982). Estrogens and PGE_2 are known to stimulate uterine blood flow in cattle (Roman-Ponce et al., 1978; Thatcher et al., 1984b; Chapter 3). Elevated luminal concentrations of estrogen and PGE_2 in the gravid uterine horn (Bartol et al., 1981; Ford, 1982; Lewis et al., 1982) and evidence supporting synthesis of estrogens (Shemesh et al., 1979; Gadsby et al., 1980; Eley et al., 1983; Chapter 2) and PGE_2 (Lewis et al., 1982) by bovine conceptuses during early pregnancy support a role for these conceptus products in pregnancy-associated elevations in uterine blood flow. Recent reports by Ford (1982) and Ford and Reynolds (1983) suggested that hydroxylated metabolites of estrogens viz., catecholestrogens, may mediate uterine blood flow responses to follicular-(estrus) and conceptus-derived (early pregnancy) E_2. They propose that an antagonistic interaction of catecholestrogens with uterine vascular α-adrenergic receptors promote reduced vascular contractility and facilitate elevated rates of uterine blood flow. Additionally, increased endometrium vascular permeability was suggested in pregnant ovine uteri as evidenced by positive Pontamine Blue dye uptake (Boshier, 1970). Local induction of uterine blood flow and vascular permeability by the conceptus may increase transfer

efficiency of luteotropic/luteal protective substances from the pregnant uterus to the CL (Reynolds et al., 1983; Thatcher et al., 1984b, 1985). However, evaluation of tritiated $PGF_{2\alpha}$ uptake in bovine endometrium and uteroovarian vascular components suggested that $PGF_{2\alpha}$ permeability may be decreased during early pregnancy (Thatcher et al., 1984b). Tissue samples, viz., endometrium, ovarian vein and ovarian artery, were collected on day 17 of pregnancy or the estrous cycle from the side ipsilateral to the CL-bearing ovary. Data demonstrated reduced $PGF_{2\alpha}$ permeability in pregnant versus cyclic tissues on day 17. Furthermore, ovarian vein permeability to $PGF_{2\alpha}$ was less than ovarian artery in pregnant cows, however, this relationship was reversed in vascular samples collected on day 17 of the estrous cycle. In pregnant tissues, responses suggest that conceptus induced reductions in vessel $PGF_{2\alpha}$ permeability were greater with regard to vessel proximity to the pregnant uterus. It may be hypothesized that conceptus products responsible for reduced $PGF_{2\alpha}$ permeability would be more concentrated in the uterine venous drainage (ovarian vein) versus ovarian artery, thus resulting in the responses observed (Thatcher et al., 1984b). Further studies are required to determine if this pregnancy-associated reduction in tissue permeability is specific for $PGF_{2\alpha}$. For example, elevated $PGF_{2\alpha}$ and PGE_2 contents of uterine flushings from pregnant versus cyclic cattle (Bartol et al., 1981; Lewis et

al., 1982) may suggest sequestration within the pregnant uterine lúmen.

Another strategy by which the conceptus maintains CL function involves attenuation of $PGF_{2\alpha}$ production by the uterine endometrium. In one of several studies, reviewed by Thatcher et al. (1984a, b; 1985), in vitro production of $PGF_{2\alpha}$ by day 17 pregnant endometrial explants was reduced by more than 50% of $PGF_{2\alpha}$ produced by day 17 cyclic endometrial explant cultures. Production of PGE_2 was similar for pregnant and cyclic endometrium. Endometrial prostaglandin synthesis was stimulated following addition of exogenous arachidonic acid to cultures, however, relative proportion of prostaglandins ($PGF_{2\alpha}$, PGFM and PGE_2) within and between pregnancy status were unchanged. These data support an antiluteolytic effect of the bovine conceptus on endometrial $PGF_{2\alpha}$ production. Levels of arachidonic acid cascade which appear to be modified during early pregnancy involve phospholipase A_2 (i.e., exogenous arachidonic acid stimulated prostaglandin synthesis) and cyclooxygenase (i.e., $PGF_{2\alpha}$ production was attenuated in pregnant endometrium with and without the addition of arachidonic acid). Although PGE_2 production did not differ between pregnancy status, it is clear that the ratio of PGE_2:$PGF_{2\alpha}$ must become elevated with suppression of $PGF_{2\alpha}$ production during early pregnancy. Such an increase in the PGE_2:$PGF_{2\alpha}$ ratio may be of physiological importance. In vivo evaluation of temporal

$PGF_{2\alpha}$ episodes in the ovarian arterial supply of cows from days 17 to 20 of the estrous cycle or pregnancy (Thatcher et al., 1984b) supported in vitro results demonstrating a reduction in $PGF_{2\alpha}$ during pregnancy. No differences were observed in frequency of $PGF_{2\alpha}$ episodes between pregnancy status. However, plasma concentrations of $PGF_{2\alpha}$ during episodic release were attenuated dramatically in pregnant cattle.

Peripheral patterns of PGFM concentrations were used as an index of uterine $PGF_{2\alpha}$ episodic production during the bovine estrous cycle (Peterson et al., 1975; Kindahl et al., 1976), early pregnancy (Kindahl et al., 1976), and following embryo transfer on day 16 post-estrus (Betteridge et al., 1984). Luteal regression, during the estrous cycle and following embryonic mortality, as determined by declining plasma P_4 concentrations, was always associated temporally with three to five large episodes of PGFM. Conversely, PGFM episodes are reduced or completely eliminated in early pregnancy. Similar observations were also reported during the estrous cycle and early pregnancy in buffaloes (Batra and Pandey, 1983). The 11-ketotetranor metabolites of $PGF_{2\alpha}$ have been evaluated recently in 24 hour, pooled urine samples of cyclic and pregnant cattle (Harvey et al., 1984; Plante et al., 1984). Although detection of acute patterns of uterine prostaglandin production are not possible using this system, clear elevations of 11-ketotetranor $PGF_{2\alpha}$ are

temporally associated with a decline in plasma P_4 concentrations during luteolysis. Peaks of the urinary metabolite are absent during early pregnancy, however, basal concentrations begin a gradual increase as pregnancy proceeds beyond 18 to 20 days. Similarly, day 17 pregnant heifers had higher basal concentrations of PGFM than nonpregnant heifers (Williams et al., 1983). It is probable that conceptus prostaglandin biosynthesis during early pregnancy contributes to elevated urinary and plasma metabolites of $PGF_{2\alpha}$. Conceptus prostaglandin production increases with gestational age in cattle (Lewis et al., 1982). It should be noted, however, that elevations in baseline prostaglandin concentrations during early pregnancy are not characteristic of luteolytic pulses detected during CL regression (Thatcher et al., 1984a). Additional support for conceptus-derived anti-luteolytic effects in cattle was reported following E_2-induction of uterine $PGF_{2\alpha}$ production on day 18 of the estrous cycle, and day 18 and 20 of pregnancy. The capacity of the uterus to produce $PGF_{2\alpha}$ (peripheral plasma PGFM) in pregnant cattle was reduced significantly from day 18 cyclic cattle responses (Thatcher et al., 1984b). Reduction in E_2-induced PGFM response was more dynamic in day 20 versus day 18 pregnant cattle suggesting that as conceptus elongation proceeds, progressively larger regions of endometrium become exposed to conceptus antiluteolytic products, resulting in a graded

decline in total uterine capacity to produce $PGF_{2\alpha}$. Additionally, duration of endometrial exposure to conceptus antiluteolytic signals may be important in this regard.

Inhibition or reduction of uterine $PGF_{2\alpha}$ production and secretory patterns may be the primary mechanism by which the developing bovine conceptus ensures CL maintenance during early pregnancy. Luteotropic and luteal protective agents, permeability and blood flow alterations may act as secondary or support systems to further reduce, dilute, or otherwise protect CL from luteolytic effects of the uterus. This argument is based on the inability of putative luteotropic and luteal protective signals to maintain CL function for extended periods in cyclic cattle. In contrast, recent reports in cattle and sheep provide evidence that antiluteolytic proteins of conceptus origin significantly extend CL lifespan and function.

Data evaluating in vitro protein synthesis and secretion by elongating conceptuses are available for cattle (Masters et al., 1982; Bartol et al., 1985), sheep (Godkin et al., 1982b; 1984a) and swine (Godkin et al., 1982a). It becomes evident from these studies that qualitative and quantitative changes in array of proteins produced are related to stage of conceptus development. In all three species, total conceptus protein production increases with gestational age (Godkin et al., 1982a, b; Bartol et al., 1985). This trend is similar to data reported for bovine

conceptus steroid (Shemesh et al., 1979; Chenault, 1980; Gadsby et al., 1980; Eley et al., 1983; Chapter 2) and prostaglandin (Lewis et al., 1982) biosynthetic activity. Additionally, apparent total protein production per mg of conceptus wet weight, in cattle, becomes elevated as rapid trophectodermal elongation is initiated between days 16 and 17 of gestation (Knickerbocker et al., 1984; Chapter 4). During this period of conceptus development, a distinct, but similar family of low Mr, acidic polypeptides constituted a major portion of proteins secreted into medium (cattle: Mr 22-26,000, pI 5.6-6.5; Bartol et al., 1985; sheep: Mr 17-21,000, pI 5.5; Godkin et al., 1982b; Martal et al., 1984b; swine: Mr 20-25,000, pI 5.6-6.2; Godkin et al., 1982a). Transient conceptus production of low Mr polypeptide species during respective periods of conceptus elongation and maternal recognition of pregnancy in cattle, sheep and swine suggest that these products may be involved in early pregnancy maintenance. Experimental evidence in the cow and ewe support this hypothesis.

Rowson and Moor (1967) demonstrated that intrauterine administration of day 14 to 15 ovine conceptus homogenates prolonged estrous cycles in ewes. However, no effect on estrous cycle length was observed following intrauterine administration of day 25 ovine conceptus homogenates or heat treated, day 12 to 14 ovine conceptuses. Similarly, introduction of homogenates or extracts of day 14 to 16

ovine conceptuses into the uterine lumen resulted in prolonged CL function and estrous cycles in eight of 12 ewes (Martal et al., 1979). Only one of six ewes exhibited prolonged luteal function and interestrous interval when day 21 to 23 conceptus homogenates were administered. Pronase or heat pretreatment completely eliminate the ability of day 14 to 16 conceptus homogenates to extend CL function in the cyclic ewe. Subsequent characterization of protein production patterns by elongating bovine (days 16 to 24; Bartol et al., 1985), ovine (days 13 to 21; Godkin et al., 1982b), and porcine (days 10.5 to 12; Godkin et al., 1982a) conceptuses verified transient biosynthesis and secretion of major, low Mr polypeptides during early pregnancy. Collectively, these data imply that biologically active proteins of conceptus origin are involved in processes leading to maternal recognition of pregnancy.

Recent data demonstrated that pooled conceptus secretory proteins (CSP), collected from medium of cultured bovine (days 16 to 18; Chapter 4) and ovine (days 15 and 16; Godkin et al., 1984b) conceptuses, substantially extend CL function and interestrous interval following intrauterine administration to cyclic cattle (days 15 through 21) and sheep (days 12 through 21), respectively. In addition, intrauterine administration of the prominent, low Mr, acidic polypeptide, termed ovine trophoblast protein-1 (oTP-1; Godkin et al., 1984b), also extended luteal function in

cyclic ewes. Thus, oTP-1 may be a primary protein signal responsible for luteal maintenance in sheep. Conceptus protein signals probably act locally at the level of uterine endometrium. For example, immunohistochemical data suggest that oTP-1 is secreted by extraembryonic trophectoderm of the ovine conceptus and binds specifically to receptors localized on uterine endometrial epithelium (Godkin et al., 1984a). In the same report, oTP-1 was shown to stimulate secretion of specific endometrial proteins in culture. In cattle (Chapters 4 and 5) and sheep (Fincher et al., 1984; Fincher, 1984), pooled CSP dramatically reduced spontaneous uterine $PGF_{2\alpha}$ episodes (Chapter 4) and E_2-induced production of uterine $PGF_{2\alpha}$ (peripheral plasma PGFM response; Fincher et al., 1984; Fincher et al.; Chapter 5). Collectively, these data demonstrate a role for conceptus-derived secretory proteins as signals during early pregnancy in cattle and sheep. It is proposed that proteinaceous conceptus signals interact with the uterine endometrium such that endometrial capacity to synthesize and secrete $PGF_{2\alpha}$ is dampened. At this time, it is not known whether CSP antiluteolytic effects are exerted directly or mediated by some induced endometrial product (Godkin et al., 1984a). Direct antiluteolytic effects of CSP may include regulation of steroid and/or oxytocin receptor populations on endometrium. In the rat, both E_2 and P_4 nuclear receptors are increased at the site of blastocyst implantation on day

5 and 6 of gestation (Logeat et al., 1980). Findley et al. (1982) reported a decline in caruncular E_2 receptors associated with side of conceptus on day 15 of pregnancy in ewes. Additionally, caruncular and intercaruncular endometrial production of protein in pregnant ewes was greater than nonpregnant endometrium. A reduction in endometrial E_2 receptors may prevent E_2-induction of oxytocin receptors and subsequent activation of $PGF_{2\alpha}$ synthesis (McCracken et al., 1984). Replenishment of endometrial P_4 receptors could also result in decreased nuclear accumulation of E_2 receptor. In this light, Fincher et al. (1984) demonstrated that oxytocin-induced uterine $PGF_{2\alpha}$ production in E_2 primed ewes was decreased significantly by intrauterine CSP administration.

Thatcher et al. (1984b) reported that day 17 pregnant endometrial explants produced less $PGF_{2\alpha}$ than cyclic endometrium in the presence or absence of exogenous arachidonic acid. They suggested that both phospholipase A_2 and cyclooxygenase activity was reduced during early pregnancy. Wlodawer et al. (1976) and Shemesh et al. (1981, 1984) reported the presence of prostaglandin inhibitors in bovine endometrium and placental tissues, respectively. It is possible that induction of such inhibitors during early pregnancy may involve conceptus protein signals. In such a scheme, endometrial $PGF_{2\alpha}$ biosynthesis may be selectively inhibited (possibly at the level of phospholipase A_2 and/or

cyclooxygenase) without influencing prostaglandin production by the conceptus (Thatcher et al., 1985).

In cattle, low molecular weight conceptus polypeptides, described by Bartol et al. (1985), may play key roles in pregnancy maintenance as was demonstrated for oTP-1 in sheep (Godkin et al., 1984a, b). Similarities in the nature and function of conceptus protein signals in cattle and sheep have been discussed. Relative to this discussion, Martal et al. (1984a) provided evidence that conceptus signals (presumably protein in nature) of cattle may be biologically active in sheep and vice-versa. Two trophoblastic vesicles (0.5 to 2 mm diameter), composed of extraembryonic trophectoderm and endoderm (embryonic disc removal), from day 13 bovine blastocysts were transferred to 11 ewes on day 12 of the estrous cycle. Two ewes exhibited prolonged luteal maintenance until at least day 38 when ewes were slaughtered. Interestingly, an elongated (>100 mm) trophoblastic vesicle was recovered from the uterus in one of these ewes, although conceptus membranes were undergoing necrosis at this time. Similarly, when two trophoblastic vesicles from day 11 to 13 ovine blastocysts were transferred to ten cyclic cattle on day 12, two heifers exhibited cycle lengths of 31 and 36 days. The authors suggested that nonspecific conceptus signals in the cow and ewe were sufficient for maintaining CL function and the

biologically active molecules responsible for CL maintenance in these species may be very similar.

As discussed earlier in this review, conceptus production of biologically active signals is closely synchronized with conceptus elongation during maternal recognition of pregnancy. Furthermore, precise synchronization between development of the conceptus and maternal uterine milieu are essential for successful membrane elongation and signal emission by the conceptus. Immunological rejection of transferred interspecie conceptus membranes undoubtedly discourages this process (Allen, 1979; Beer and Billingham, 1979; Rossant et al., 1982; Wielen and King, 1984). In this light, observed extension of CL function in 20% of cattle and sheep receiving interspecific trophoblastic vesicle transfers (Martal et al., 1984a) seems even more remarkable. Recent data (S.D. Helmer, W.W. Thatcher, F.W. Bazer, P.J. Hansen and R.M. Roberts, 1984, unpublished observations; see Thatcher et al., 1985) lend additional support that conceptus antiluteolytic protein signals may be similar in cattle and sheep. Antibodies developed against oTP-1 (Godkin et al., 1984a) were used in an ouchterlony double immuno-diffusion system with unfractionated bovine conceptus secretory proteins. Two precipitin bands were formed against unfractionated bovine coneptus secretory proteins, one of which showed partial identity with either purified oTP-1 or unfractionated ovine

conceptus secretory proteins. Subsequently, an immunoprecipitate of tritiated bovine conceptus secretory protein fraction which crossreacted with oTP-1 antiserum was solubilized and analyzed by polyacrylamide gel electrophoresis and fluorography. Immunoprecipitated bovine conceptus secretory proteins were detected in the 20,000 and 22,000 Mr range. Collectively, these data suggest that analogous mechanism of "Maternal Recognition of Pregnancy, possibly involving the low Mr, acidic polypeptide species, may exist in cattle and sheep.

The conceptus may also exert an indirect antiluteolytic effect as a result of subtle suppression and acceleration in folliculogenesis and atresia, respectively (Dufour et al., 1984). Such a conceptus interaction may be important since exogenous estrogens have been shown to stimulate uterine $PGF_{2\alpha}$ production in cattle (Chapter 3) resulting in luteal regression (Greenstein et al., 1958; Wiltbank, 1966; Eley et al., 1979). Estrogens may also act directly on the CL to antagonize LH stimulated P_4 production (Williams and Marsh, 1978). Conversely, CL function was extended following destruction of ovarian follicles during the luteal phase of the bovine estrous cycle (Villa-Godoy et al., 1981). Thus, conceptus regulation of follicular development and hence, estrogen production may complement the intrauterine antiluteolytic efforts of the conceptus to suppress uterine $PGF_{2\alpha}$ synthesis during early gestation.

In conclusion, events associated with "Maternal Recognition of Pregnancy" reflect dynamic as well as subtle alterations in maternal and conceptus physiology. Numerous putative conceptus signals have been identified, although their precise roles in "Maternal Recognition of Pregnancy" are just beginning to be understood. Maintenance of CL function and an embryotrophic uterine milieu during early pregnancy is accomplished by several interdependent, conceptus-mediated strategies. Evaluation and clarification of the complex interactions between conceptus and its maternal host presents a considerable and exciting task for the future.

CHAPTER 2
PATTERNS OF PROGESTERONE METABOLISM BY DAYS 19-23 BOVINE CONCEPTUS AND ENDOMETRIAL EXPLANTS

Introduction

Maternal recognition of pregnancy (Short, 1969) in cattle occurs by day 16 (Betteridge et al., 1980; Northey and French, 1980; Dalla Porta and Humblot, 1983). During this period the bovine conceptus initiates a phase of rapid elongation (Hawk et al., 1955; Greenstein and Foley, 1958b; Betteridge et al., 1980) and elevated endocrine activity (Shemesh et al., 1979; Chenault, 1980; Lewis et al., 1982; Eley et al., 1983; Bartol et al., 1985). Conceptus endocrine products, in turn, direct events which are essential for the establishment and maintenance of pregnancy. Biosynthesis and possible mechanisms of action for conceptus-derived prostaglandin and protein signals have been discussed earlier (see: Literature Review). Recent reports demonstrate the capacity for synthesis and metabolism of various steroids by elongating bovine conceptuses (Shemesh et al., 1979; Chenault, 1980; Gadsby et al., 1980; Eley et al., 1983). Based on these studies it is clear that small amounts of estrogen biosynthesis results following conceptus incubation with androgen precursors

(androstenedione, A_4; testosterone, T; dehydroepiandrosterone, DHA). In addition, Shemesh et al (1979) reported detectable amounts of immunoreactive P_4, T and estradiol-17β (E_2) in some bovine conceptus extracts on days 15 and 16 of gestation. Total content of these steroids were elevated in medium following 48 hour conceptus cultures in the absence of exogenous precursors. Thus the bovine conceptus may utilize C21 steroids, viz., P_4 and pregnenolone, as precursors for androgen and estrogen production. Conversely, Eley et al. (1983) were unable to demonstrate tritiated-P_4 (10 ng) conversion to estrogens when shorter culture periods (3 to 6 hours) and less sensitive procedures of identification (liquid column chromatography and recrystallization) were utilized. Currently, no data are available with regard to relative ratios of estrone (E_1), E_2 and estriol (E_3) production by the early bovine conceptus.

Indirect physiological data support conceptus estrogen biosynthesis during maternal recognition of pregnancy. Blood flow to the gravid uterine horn is transiently elevated between days 14 and 18 of gestation in cattle (Ford et al., 1979; Ford and Chenault, 1981). Uterine blood flow during the bovine estrous cycle and early pregnancy are highest during periods of low P_4:E_2 ratios in plasma. Furthermore, exogenous estrogens stimulate uterine blood flow in cyclic cattle (Roman-Ponce et al., 1978; Chapter 3).

The bovine conceptus possesses an extremely active 5β-reductase enzyme system (Chenault, 1980; Eley et al., 1983) whereas the endometrium produces primarily 5α-reduced steroids (Eley et al., 1983). Metabolites identified were hydroxylated in an α-configuration at the 3 and/or 17 (A_4 substrate) and 20 (P_4 substrate) positions. Roles of 5β-reduced steroids during early pregnancy in cattle have not been studied.

It was the objective of this study to characterize patterns of P_4 metabolism to 5β/α-reduced products and estrogens by bovine conceptus and endometrial tissues during early pregnancy and to identify major conceptus metabolites for future evaluation of physiological roles during early pregnancy (Chapter 4).

Materials and Methods

Materials

Minimum essential medium (MEM; GIBCO, Grand Island, NY) supplemented with non-essential amino acids (GIBCO, Grand Island, NY), antibiotic/antimycotic (GIBCO, Grand Island, NY), 200 units of insulin/L (Sigma Chemical Company, St. Louis, MO) and 1 g glucose/L (Fisher Scientific, Orlando, FL) was used for rinsing and incubating conceptus and endometrial tissues. All radiolabelled steroids were purchased from New England Nuclear, Boston, MA. Tritiated

progesterone (P_4; [1,2,6,7-^{3}H(N)], 97 Ci/mmole) was used as substrate for tissue metabolism. Tritiated 5β-pregnan-3α,20α-diol ([1,2-^{3}H(N)], 55 Ci/mmole) was utilized as a radiolabelled marker to examine elution of this steroid with respect to conceptus steroid metabolites in high pressure liquid chromatography (HPLC) systems. Carbon-14-labelled marker steroids used were androstenedione (A_4; [4-^{14}C], 57.4 mCi/mmole), testosterone (T; [4-^{14}C], 57 mCi/mmole), estradiol-17β (E_2; [4-^{14}C], 57 mCi/mmole) and estrone (E_1; [4-^{14}C], 57 mCi/mmole). Radiolabelled steroids were judged to be greater than 95% pure based on liquid chromatography or HPLC trials.

Several radioinert steroids (P_4, A_4, T, E_1, E_2, estriol, 5α/5β-androstane-3,17-dione, 5α/5β-pregnane-3,20-dione, 3α-hydroxy-5β-pregnan-20-one) were used as markers for unknown, tritiated P_4 metabolite identification in HPLC and gas/liquid chromatography (GLC) systems. All radioinert steroids were purchased from Steraloids, Wilton, NH, with the exception of 3α-hydroxy-5β-pregnan-20-one, which was a generous gift from Dr. John R. Chenault of the Upjohn Company, Kalamazoo, MI. Purity of radioinert steroids was verified by HPLC and GLC analyses. All reagent grade solvents (Fisher Scientific, Fair Lawn, NJ; Eastman Kodak, Rochester, NY) were distilled before use. Acetonitrile (HPLC grade; Fisher Scientific, Fair Lawn, NJ) and various

laboratory grade chemicals were used as received. Double distilled-deionized water was prepared in the laboratory.

Animals and Tissue Collection

Angus cows (n=11) were serviced naturally at observed estrus (day 0) by an intact Brown Swiss bull and slaughtered on days 19 (n=5), 21 (n=1), 22 (n=1) or 23 (n=4) post-mating. Reproductive tracts were excised following exsanguination, sealed in a plastic bag, and placed on ice while being transported to the laboratory. Uterine horns were trimmed of excess tissue, and ovaries and oviducts removed. Serosal and myometrial tissue layers of both horns were gently peeled back along the mesometrial border until the outside of the endometrial layer was visable. Care was taken not to crush the uterine-contained conceptus or expose the uterine lumen during this procedure. When the uterine lumen was opened, conceptus tissue was removed with sterile forceps. Conceptuses (n=11) were washed in 15 ml of culture medium (MEM) before being transferred to culture flasks. Endometrial tissue was collected from eight of these cattle on days 19 (n=3), 21 (n=1) and 23 (n=4) of gestation. Endometrium was dissected from both uterine horns with Metzenbaum (curved) scissors and minced into 1 to 3 mm^3 pieces. Minced endometrial tissue was rinsed in 15 ml of MEM and approximately 500 mg (497 ± 8.1 mg) transferred to culture flasks.

Uteri from an additional seven Angus cows were nonsurgically flushed with sterile Dulbeccos' phosphate buffered saline (pH 7.4; Dulbecco and Vogt, 1954) on day 19 post-mating. Conceptuses (n=7) were recovered and transferred to culture flasks within 15 minutes of collection. It should be noted that these seven cows had been injected with estradiol-17β (E_2; 3 mg I.V.) on day 18 of pregnancy as part of another experiment (Thatcher et al., 1984b). Conceptus metabolic results relevant to this point are discussed later.

Tissue Incubations and Extraction of Steroids

Approximately 20 ng of $[^3H]$-P_4 in organic solvent were aliquoted into 25 ml flasks. Solvents were evaporated under N_2 gas, 3 ml of warm (37 C) MEM were added to each flask and contents vortexed for several minutes. Whole conceptuses and minced endometrial tissue (Table 2-1) were weighed and placed individually into incubation flasks, gased with O_2:CO_2 (95:5) and gently rocked in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Group, Chicago, IL) at 37 C. Incubations were stopped at the end of 3 hours with addition of 5 to 10 ml acetone to each flask. Incubation flasks and contents were stored at -20 C until steroid extraction. Approximately 5000 counts per minute (cpm) of $[^{14}C]$-P_4, $[^{14}C]$-T and $[^{14}C]$-E_1 markers were added to flasks to account for procedural losses during extraction and chromatography. Incubation flask contents were

Table 2-1. Tissue incubations

Day	Endometrium Incubations	(no. cows)	Mean ± S.E.M. Wet weight (mg)
19	5	(3)	499.5 ± 16.7
21-23	13	(5)	496.0 ± 9.2
TOTAL	18	(8)	497.0 ± 8.1

Day	Conceptus Incubations	(no. cows)	Mean ± S.E.M. Wet weight (mg)*
19	12	(12)	265.0 ± 22.5
21-23	6	(6)	358.0 ± 5.4
TOTAL	18	(18)	296.0 ± 18.9

* Pieces of conceptus tissue recovered from days 19 (n=2) and 21 (n=1) pregnant cattle were not included in the wet weight calculations; wet weights for these tissues were: 46.5 ± 2.5 and 53 mg, respectively.

transferred to individual, 50 ml, stainless steel centrifuge tubes. Flasks were rinsed thrice with 10 ml of acetone, each rinse being deposited into the appropriate sample tubes. Tissues were then homogenized with a Brinkman polytron (Westbury, NY). The polytron shaft was cleaned of tissue fragments and rinsed (10 ml acetone) into sample tubes. Samples were centrifuged at 18,000 x g for 20 minutes. Supernatant (5 ml MEM plus 40 ml acetone) was poured into 125 ml separatory funnels. Tissue pellets were reextracted from one to three times with 10 ml of acetone each and centrifuged. Supernatants following reextraction were combined with previous acetone extracts. Acetone was evaporated under N_2 gas leaving an aqueous MEM volume of 3 ml in each separatory funnel. Thirty milliliters of methylene dichloride was added to each MEM fraction and vigorously agitated for several minutes. Following complete separation of the MEM (top layer) and methylene dichloride (bottom layer) phases, the methylene dichloride fraction was drained into glass conical tubes, dried under N_2 gas, and stored at -20 C until liquid-column chromatography. Aqueous MEM was collected into glass vials and 100 μl counted for radioactivity.

<u>Liquid-Column Chromatography</u>

Sephadex LH-20 (Sigma Chemical Company, St. Louis, MO) column chromatography was employed for initial separation of conceptus and endometrial steroid extracts. The solvent

system consisted of a cyclohexane (CyH), benzene (Bz) and methanol (MeOH) mixture (90:25:5). Column dimensions were 12 x 1.5 cm. Procedures for preparation of Sephadex LH20 and pouring of columns are discussed in Appendix A.

Steroid extracts were reconstituted in 0.5 ml of CyH:Bz:MeOH (90:25:5) and loaded carefully on the top of the Sephadex LH20 bed. Collection of 1 ml fractions was begun at this point. Sample containers were rinsed with an additional 0.5 ml of the solvent mixture and applied to the column immediately after the first extract had moved into the column bed. A 1 ml solvent 'cushion' was then loaded after which the glass column was filled with solvent mixture and a 300 ml solvent reservoir placed in line with the column to maintain a constant head pressure. Column flow rates were adjusted and maintained between 6 and 8 drops per minute (1 drop per 7 to 9 seconds). Eighty 1 ml fractions were collected using an automated fraction collector (Model 328, ISCO, Lincoln, NE) followed by a single 40 ml fraction collected at the end of each 80 ml run. One hundred microliters of each 1 ml fraction was counted to evaluate radioactivity elution profiles. Columns were washed with 50 to 100 ml of CyH:Bz:MeOH (90:25:5) between each sample run.

Steroid metabolites were divided into eight areas based on peaks of tritiated radioactivity (Eley et al., 1983) and relative elution to [^{14}C]-markers (P_4, T, E_1).

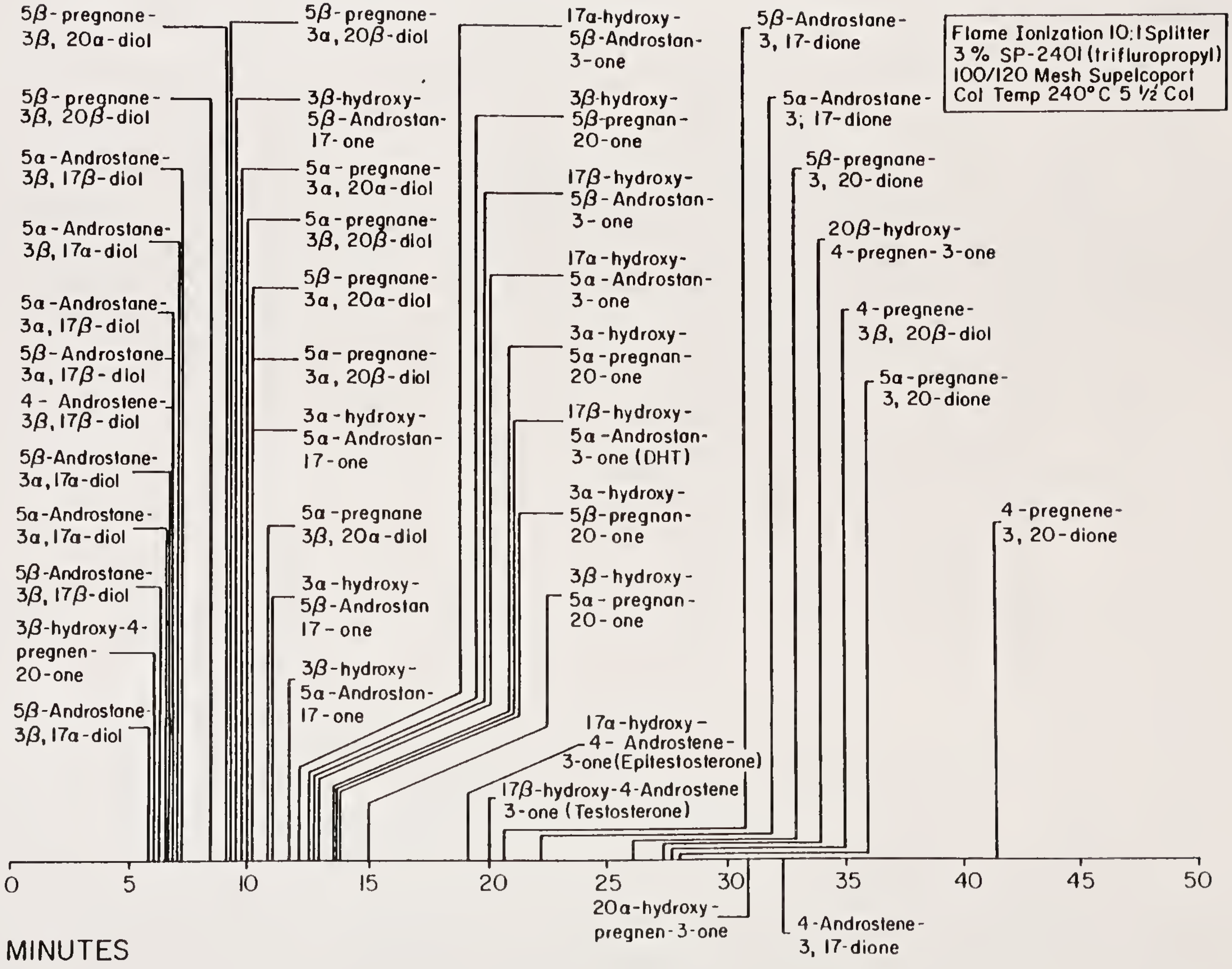
Gas-Liquid Chromatography (GLC)

A Varian, model 2740, gas-liquid chromatograph (Palo Alto, CA) was utilized to further separate and identify radiolabelled metabolites. The column used was a 168 cm Supelcoport, coiled glass column packed with 3% trifluoropropyl silicone on a Supelcoport 100/120 mesh (SP-2401; Bellefonte, PA). Elution of radioinert steroids were registered on a strip chart recorder following 10:1 sample splitting and flame ionization detection. Nitrogen gas pressure was maintained at 26.5 p.s.i., and hydrogen and air flow rates were 33 and 300 ml/minute, respectively. Temperatures for the column, injector and detector were 240, 262 and 265 C, respectively.

Prior to running samples on GLC, elution patterns of 42 radioinert steroids (Steroid Reference Collection, National Research Council, London, England) were determined (Figure 2-1) to aid in the identification of unknown conceptus metabolites. Steroid elution times and mobilities relative to P_4 (R_f/P_4) are listed in Appendix B.

Approximately 5000 cpm of tritiated radioactivity in single conceptus (n=3) areas 2, 3 and 5 (Sephadex LH20) were solubilized in carbon disulfide and injected separately on GLC with radioinert standards. One minute fractions were collected by condensing radiolabelled compounds in cold, 'U'-shaped, glass collection tubes inserted over the column exit port. Collection tubes (I.D., 2 mm) consisted of a 10

Figure 2-1. Retention times for radioinert steroids using gas liquid chromatography. Steroids were donated from the Steroid Reference Collection, International Research Council, London, England.



5β-pregnane-3β, 20α-diol
5β-pregnane-3β, 20β-diol
5α-Androstane-3β, 17β-diol
5α-Androstane-3β, 17α-diol
5α-Androstane-3α, 17β-diol
5β-Androstane-3α, 17β-diol
4-Androstene-3β, 17β-diol
5β-Androstane-3α, 17α-diol
5α-Androstane-3α, 17α-diol
5β-Androstane-3β, 17β-diol
3β-hydroxy-4-pregnen-20-one
5β-Androstane-3β, 17α-diol
5β-pregnane-3α, 20β-diol
3β-hydroxy-5β-Androstan-17-one
5α-pregnane-3α, 20α-diol
5α-pregnane-3β, 20β-diol
5β-pregnane-3α, 20α-diol
5α-pregnane-3α, 20β-diol
3α-hydroxy-5α-Androstan-17-one
5α-pregnane 3β, 20α-diol
3α-hydroxy-5β-Androstan 17-one
3β-hydroxy-5α-Androstan-17-one
17α-hydroxy-5β-Androstan-3-one
3β-hydroxy-5β-pregnan-20-one
17β-hydroxy-5β-Androstan-3-one
17α-hydroxy-5α-Androstan-3-one
3α-hydroxy-5α-pregnan-20-one
17β-hydroxy-5α-Androstan-3-one (DHT)
3α-hydroxy-5β-pregnan-20-one
3β-hydroxy-5α-pregnan-20-one
17α-hydroxy-4-Androstene-3-one (Epitestosterone)
17β-hydroxy-4-Androstene 3-one (Testosterone)
20α-hydroxy-pregnen-3-one
5β-Androstane-3, 17-dione
5α-Androstane-3; 17-dione
5β-pregnane-3, 20-dione
20β-hydroxy-4-pregnen-3-one
4-pregnene-3β, 20β-diol
5α-pregnane-3, 20-dione
4-Androstene-3, 17-dione
4-pregnene-3, 20-dione
Flame Ionization 10:1 Splitter
3 % SP-2401 (trifluropropyl)
100/120 Mesh Supelcoport
Col Temp 240°C 5 ½' Col
0
5
10
15
20
25
30
35
40
45
50
MINUTES

cm straight section followed by a 15 cm loop. Collection tubes were rinsed with 5 ml acetone into glass scintillation vials. Acetone was evaporated under N_2 gas, 3 ml scintillation fluid added to each vial, and vials counted for radioactivity. Radioactivity elution patterns were then collated with radioinert steroid chromatograms. Identical procedures were used to identify conceptus (n=7) and endometrial (n=4) steroid products following chromic trioxide (CrO_3) oxidation (Bush, 1961) of steroids in pooled areas 1 through 6 (Sephadex LH20).

High Performance Liquid Chromatography (HPLC)

Further identification and verification of conceptus and endometrial steroid metabolites were accomplished on HPLC. Two HPLC systems were used during estrogen determinations: 1) Waters Autosampler (WISP-710B; Waters Associates Inc., Milford, PA) and variable UV wavelength detector (275-280 λ; model 450; Waters Associates Inc., Milford, PA); 2) Perkin Elmer Series 4 Liquid Chromatograph and Spectrophotometric Detector (275-280 λ; model LC-85; Perkin Elmer Inc., Norwalk, CT). Identical octadecylsilica (ODS) columns (Radial Pack, C-18, 10 μ; Waters Associates Inc., Milford, PA) were used in both systems. Solvent mixtures used in systems 1 and 2 were acetonitrile:H_2O (45:55) and acetonitrile:H_2O (55:45), respectively (Table 2-8). Estrogen fractions from Sephadex LH20 column

chromatography were pooled (viz., area of $[^{14}C]$-E_1 elution to end of column run) from single conceptus (n=9) and endometrial (n=10) samples. Approximately 10,000 cpm of tritium and 200 to 500 cpm of $[^{14}C]$-E_1 and E_2 markers were dissolved in 100 µl of acetonitrile containing radioinert E_1, E_2 and E_3 standards and injected for HPLC analysis. Injection volumes were 20 to 50 µl and solvent flow rates were between 1.5 and 2.0 ml/minute. Fractions were collected in scintillation vials on an ISCO fraction collector (model 328; Lincoln, NE) every 0.1 or 0.2 minutes; 3.5 ml of 25% Triton-X (Research Products International, Elk Grove Village, IL) scintillation fluid were added to each vial, and vials counted for radioactivity. Radioactivity ($[^3H]$ and $[^{14}C]$) was then collated with chromatograms of radioinert estrogen standards.

Production of neutral steroid metabolites were evaluated on the Perkin-Elmer HPLC system, described earlier. A 5 µ guard column (RP-18 OD-GU; Brownlee Labs Inc., Santa Clara, CA) and Perkin Elmer 3 µ octadecylsilica column (HS-3 C18; Perkin Elmer Inc., Norwalk, CT) were used for separation of steroids. An acetonitrile and water (54:46) solvent system was employed at a flow rate of 1.5 ml/minute. Fractions were collected every 0.2 minutes and evaluated as described earlier. Radioactivity in single conceptus (n=15) and endometrial (n=10) samples from Sephadex LH20 areas 1 through 6 were pooled, oxidized and

injected on HPLC with [^{14}C]-A_4 and P_4 markers, and radioinert standard steroids, viz. T, A_4, 5α/5β-androstanedione, P_4, 5β-pregnan-3α-ol-20-one and 5α/5β-pregnanedione. Similarly, radioactive metabolites were evaluated in individual and pooled conceptus samples from Sephadex LH20 areas 2 (n=15), 3 (n=15) and 5 (n=13).

Results

LH20 Column Chromatography of Conceptus and Endometrial Metabolites

Recoveries of [^{14}C] markers were calculated in 10 randomly selected samples of conceptus and endometrial incubations to evaluate procedural losses of radioactivity during extraction and LH20 column chromatography. Based on [^{14}C] radioactivity counted following LH20 chromatography, recoveries (mean ± S.E.) for P_4, T and E_1 were 91.0 ± 5.2, 76.0 ± 3.2 and 83.5 ± 3.7 percent, respectively. Mean ± S.E. elution (ml) of [^{14}C] markers was 9.7 ± 0.2 (P_4; n=37), 31.5 ± 0.8 (T; n=10) and 47.5 ± 1.3 (E_1; n=37).

Eight areas of [^{3}H] metabolite elution were defined following column chromatography (Table 2-2). No differences were observed between the metabolic patterns of endometrial incubations from day 19 versus days 21 to 23 of pregnancy (Table 2-3). Progesterone metabolism was incomplete with 35.4 ± 2.1 (n=17) percent of radioactivity remaining in the substrate peak (area 2) after 3 hours of incubation. Major

Table 2-2. Elution of progesterone metabolites with reference to [^{14}C]-estrone after sephadex LH20 column chromatography (CyH:Bz:MeOH [90:25:5])

Area[a]	N	Peak elution (ml)	Range (ml)	R_fE_1[b]
1	10	4.21	3-5	.088
2	10	9.42	8-11	.202
3	10	14.33	12-16	.305
4	10	19.80	17-24	.413
5	10	29.43	26-35	.639
6	10	38.82	36-44	.829
7	10	54.20	51-61	1.183
8	9	67.60	62-73	1.460

[a] Metabolite areas from bovine endometrium and conceptus incubations

[b] Peak elution of [^{3}H] metabolites relative to [^{14}C]-E_1; R_f for estrone (E_1) = 1.000

Table 2-3. Percent distribution of bovine endometrial [^{3}H]-progesterone metabolites following LH20 column chromatography (CyH:Bz:MeOH [90:25:5])

ENDOMETRIAL INCUBATIONS (DAY)

Area	1(19)	2(19)	3(19)	4(19)	5(19)	Day 19 $\bar{X} \pm$ S.E.	6(21)	7(21)	8(21)	9(23)
1	0	5.2	0	2.2	0	1.5 ± 1.0	21.7	14.6	5.0	5.4
2	44.2	43.8	50.3	32.9	32.6	40.8 ± 3.5	20.8	35.2	49.4	35.9
3	21.0	22.9	32.9	33.2	25.8	27.2 ± 2.5	21.8	16.4	17.5	18.2
4	14.5	13.9	14.9	16.5	21.0	16.2 ± 1.3	11.2	12.2	18.2	21.6
5	11.7	8.7	18.7	6.1	9.1	10.9 ± 2.1	12.3	9.3	7.7	9.6
6	1.4	2.1	3.3	3.5	3.5	2.8 ± 0.4	3.9	4.7	1.5	3.8
7	1.2	0.7	3.3	1.3	1.6	1.6 ± 0.4	1.0	4.3	0.2	2.4
8	5.9	3.3	9.5	4.3	5.8	5.8 ± 1.0	7.4	3.3	0.4	3.0

Area	10(23)	11(23)	12(23)	13(23)	14(23)	15(23)	16(23)	17(23)	Days 21-23 $\bar{X} \pm$ S.E.
1	1.6	3.2	1.1	6.7	14.8	1.6	1.4	4.9	6.8 ± 1.8
2	37.9	32.9	38.9	17.9	26.7	35.2	39.8	26.7	33.1 ± 2.4
3	22.3	20.7	19.8	24.5	19.7	21.1	17.4	21.1	20.0 ± 0.7
4	21.4	13.8	22.0	19.4	-	23.5	25.1	18.5	18.8 ± 1.3
5	12.4	16.9	13.1	-	-	9.7	15.3	12.8	11.9 ± 0.9
6	1.6	5.5	1.9	-	-	2.4	7.6	5.4	3.8 ± 0.6
7	0.7	1.8	0.8	-	-	0.9	5.6	5.0	2.3 ± 0.6
8	1.9	5.1	2.2	-	-	4.9	3.2	5.6	3.7 ± 0.6

percentage of P_4 metabolites eluted in areas 3 (22.1 ± 1.3; n=17), 4 (18.0 ± 1.0; n=16) and 5 (11.6 ± 0.9; n=15), with less than 10 percent residing in areas 6 through 8 (Table 2-3, Figure 2-2). In contrast, day 19 to 23 conceptus tissues actively metabolized $[^3H]$-P_4 substrate (Table 2-4, Figure 2-3) during the 3 hour incubation period. Extent of P_4 metabolism tended to be greater by day 23 (97.4 ± 0.2) versus days 21 to 22 (91.2 ± 1.3) or day 19 (88.1 ± 1.7) conceptus tissues (Table 2-4). Increased tissue mass of day 23 conceptuses (Table 2-1) may contribute to the apparent increased P_4 metabolic capacity at this stage. However, tissue metabolic activity may be increased as well since tissue fragments from two day 19 conceptuses (43 and 50 mg wet weight) and a day 21 conceptus (53 mg wet weight) metabolized 81.8 ± 1.0 versus 93 percent of available P_4 substrate, respectively (see Table 2-4, conceptus numbers 1-19, 5-19 and 10-21). Progesterone metabolic profiles were qualitatively different by day 19 versus days 21 to 23 conceptuses (Table 2-4; Figures 2-3 and 2-4). Most notable was the shift in P_4 metabolism to more polar steroids in area 6, eluting just prior to $[^{14}C]$-E_1. Chromatograms from day 19 conceptus incubations exhibited a double peak of radioactivity in area 6 containing 9.5 ± 1.0 percent of total eluted radioactivity. By day 23, area 6 contained 34.5 ± 4.7 percent of total eluted radioactivity with over 50% eluting in the earlier peak (Figure 2-4). In addition,

Figure 2-2. Sample elution pattern of tritiated endometrial (day 19) metabolites following a 3 hour incubation with tritiated progesterone. Sephadex LH20 column chromatography (CyH:Bz:MeOH [90:25:5]).

ENDOMETRIAL [^{3}H]-P4 METABOLITE ELUTION PROFILE
AFTER LH-20 COLUMN CHROMATOGRAPHY (CyH:Bz:MeOH [90:25:5])

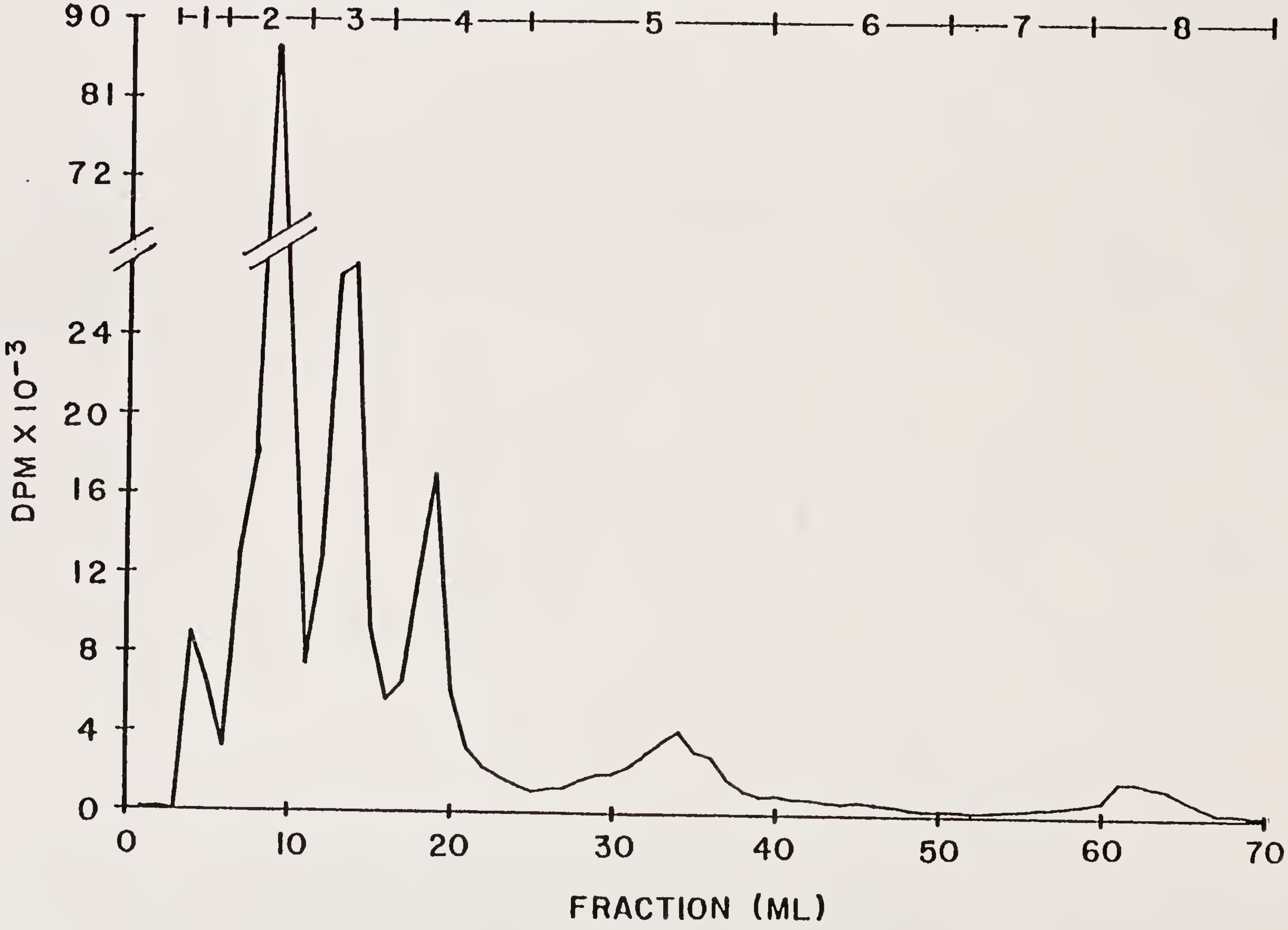


Figure 2-3. Sample elution pattern of tritiated conceptus (day 19) metabolites following a 3 hour incubation with tritiated progesterone. Sephadex LH20 column chromatography (CyH:Bz:MeOH [90:25:5]).

CONCEPTUS [^{3}H]-P4 METABOLITE ELUTION PROFILE
AFTER LH-20 COLUMN CHROMATOGRAPHY (CyH:Bz:MeOH [90:25:5])

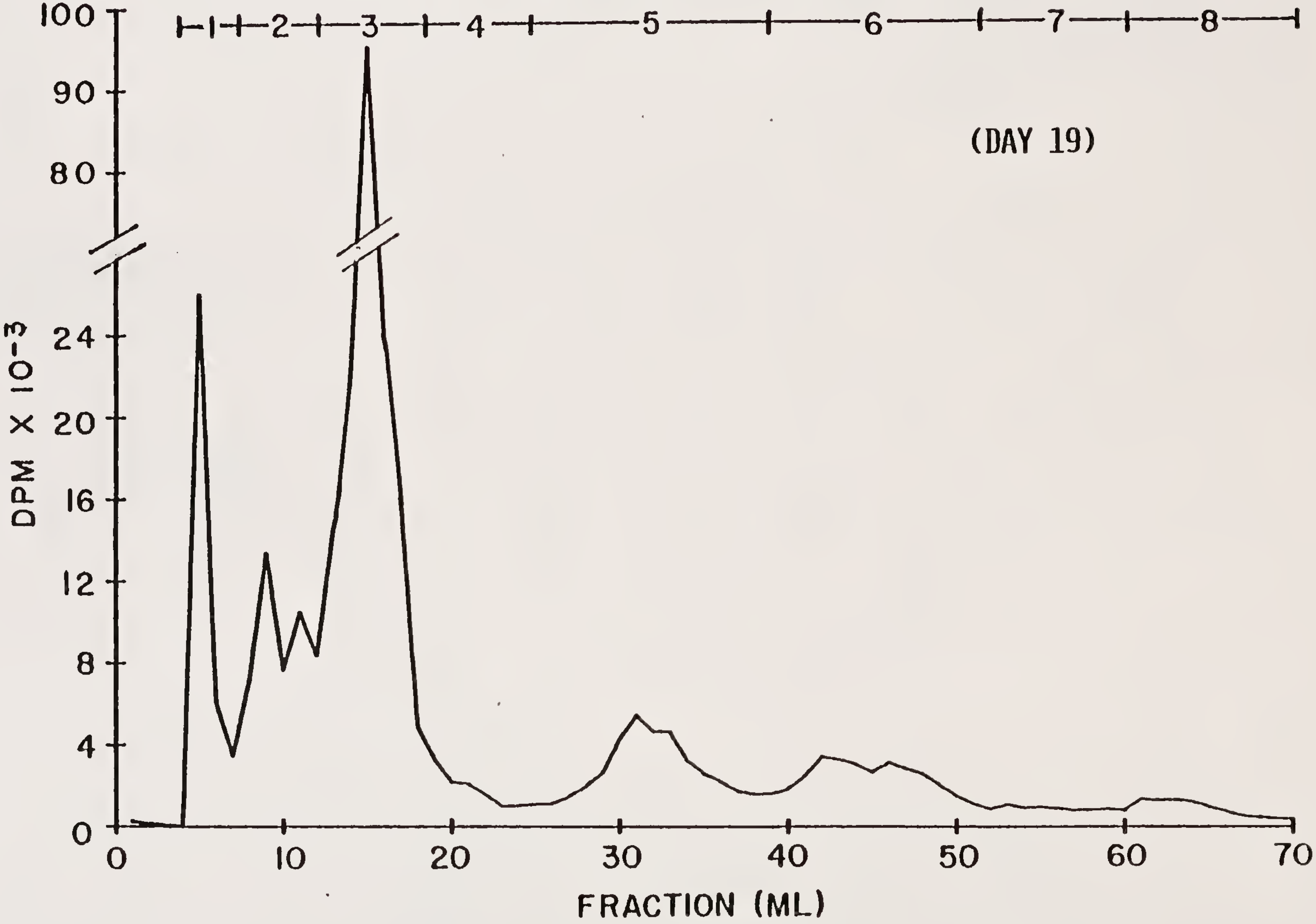


Table 2-4. Percent distribution of bovine conceptus [^{3}H]-progesterone metabolites following LH20 column chromatography (CyH:Bz:MeOH [90:25:5])

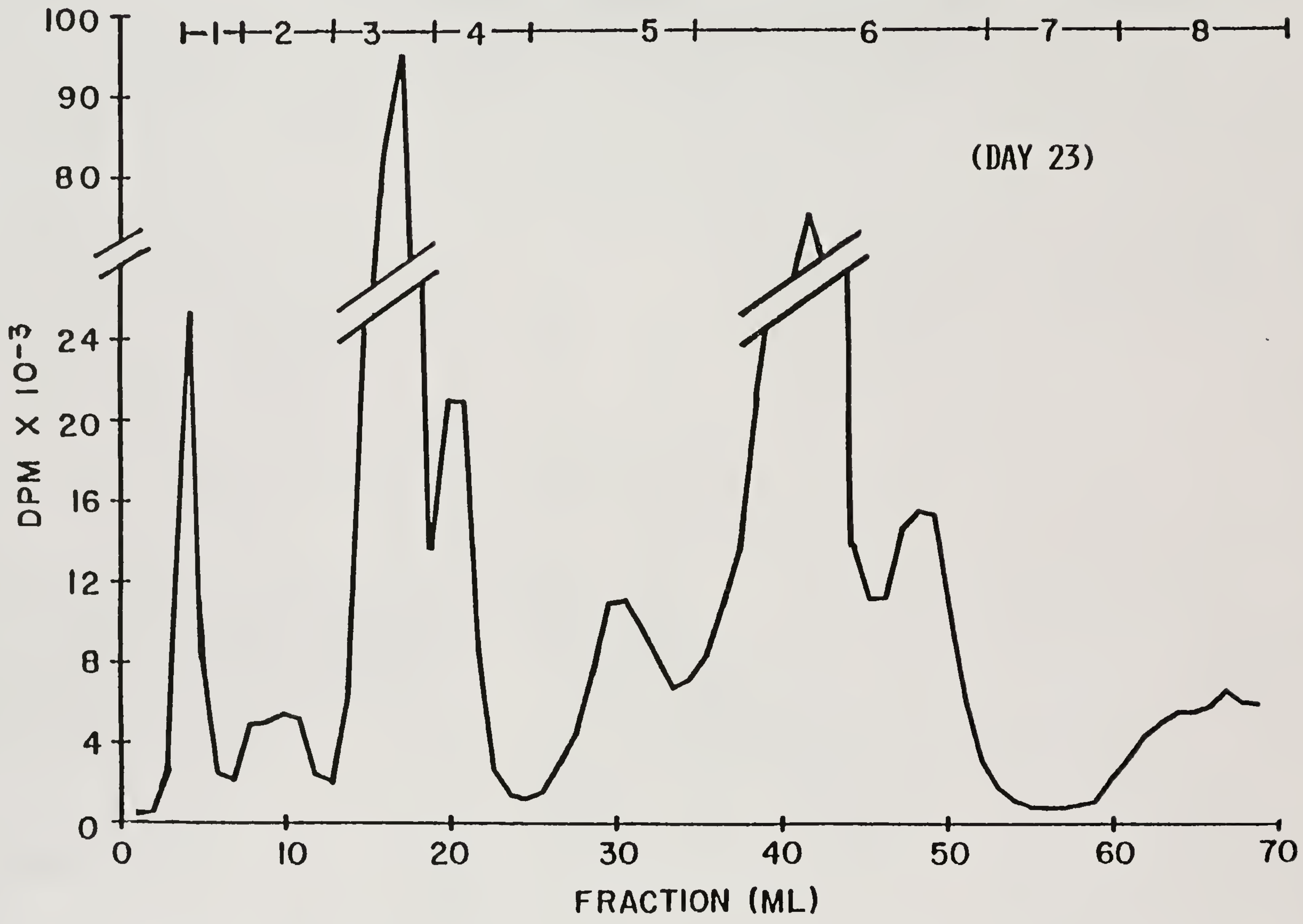
CONCEPTUS INCUBATIONS (DAY)

Area	1(19)	2(19)	3(19)	4(19)	5(19)	6(19)	7(19)	8(19)	9(19)	Day 19 $\bar{X} \pm$ S.E.
1	5.2	11.0	18.3	12.7	6.2	12.7	12.2	8.2	15.1	11.3 ± 1.3
2	19.6	7.0	13.2	12.7	16.8	15.9	9.4	9.9	2.3	11.9 ± 1.7
3	40.5	50.8	38.1	36.5	43.2	38.4	52.8	57.4	47.5	45.0 ± 2.3
4	7.8	1.5	5.4	4.4	5.5	5.4	1.4	1.6	4.1	4.1 ± .7
5	10.3	6.6	8.6	16.1	12.1	14.1	12.5	8.5	12.4	11.2 ± .9
6	9.8	12.7	9.3	13.3	6.1	8.8	5.5	6.4	13.5	9.5 ± 1.0
7	4.9	3.7	3.8	2.1	6.1	2.2	4.1	2.1	2.5	3.5 ± .4
8	1.9	1.2	3.4	2.2	4.0	2.5	1.4	1.8	2.6	2.3 ± .3

Area	10(21)	11(22)	12(23)	13(23)	14(23)	15(23)	Days 21-23 $\bar{X} \pm$ S.E.
1	5.8	3.5	9.6	3.7	8.0	9.6	7.7 ± 1.9
2	7.0	10.6	3.3	2.7	2.3	2.3	5.1 ± 1.4
3	57.9	18.6	36.5	30.9	39.0	28.8	39.6 ± 6.5
4	-	11.6	4.2	9.3	6.8	5.1	7.8 ± 0.8
5	6.1	5.8	10.5	7.0	10.1	6.7	8.7 ± 1.5
6	14.2	31.1	23.9	46.4	26.8	41.0	30.6 ± 4.4
7	4.9	11.0	3.7	1.0	0.7	0.8	3.0 ± 1.3
8	4.0	2.5	2.1	5.8	6.4	5.5	4.5 ± 0.7

Figure 2-4. Sample elution pattern of tritiated conceptus (day 23) metabolites following a 3 hour incubation with tritiated progesterone. Sephadex LH20 column chromatography (CyH:Bz:MeOH [90:25:5]).

CONCEPTUS [^{3}H]-P4 METABOLITE ELUTION PROFILE
AFTER LH-20 COLUMN CHROMATOGRAPHY (CyH:Bz:MeOH [90:25:5])



definitive peaks of radioactivity in area 4 were not observed in day 19 conceptus incubations but were consistently present following conceptus incubations on days 22 and 23.

GLC of Conceptus and Endometrial Metabolites

Base structures of conceptus (n=7) and endometrial (n=4) steroid metabolites were determined following CrO_3 oxidation and GLC analysis of pooled areas 1 through 6 (Tables 2-5 and 2-6). The oxidation procedure converted α and β hydroxyl isomers at positions 3, 17 and 20 to keto-groups. It should be noted that 17 hydroxylated progestins would be converted to either A_4 or $5\alpha/5\beta$-androstanedione following CrO_3 oxidation (Bush, 1961). Loss of the C21 side-chain was verified in our system, as A_4 resulted from oxidation of 17α-hydroxy-P_4 (data not shown). Separation of radioinert standards and their respective coeluting radioactive metabolites are depicted in figures 2-5 and 2-6. Endometrial tissues metabolized P_4 to predominantly 5α-reduced pregnane steroids which were converted to 5α-pregnanedione following CrO_3 oxidation. Small amounts of 5β-pregnanedione and A_4 were also observed after oxidation of endometrial metabolites. In agreement with results from LH20 column chromatography, P_4 was the major steroid present in oxidized endometrial samples suggesting that metabolic activity of endometrial tissue was less than that of the conceptus. In contrast to endometrial metabolism, reduction

Table 2-5. Percent distribution of radioactivity eluted with standard steroid peaks after CrO_3 oxidation and gas chromatography of endometrial metabolites

	Endometrium (Day)				
Metabolite[a]	1(19)	2(19)[b]	3(21)	4(23)	$\bar{X} \pm$ S.E.[c]
5β-A	0.17	0.30	0.15	0.50	0.27 ± 0.11
5α-A	0.43	1.00	0.30	0.80	0.51 ± 0.15
5β-P	3.00	13.00	11.10	9.10	7.73 ± 2.44
5α-P	27.10	52.70	27.20	30.80	28.37 ± 1.22
A_4	5.80	2.80	2.35	1.90	3.35 ± 1.23
P_4	41.00	18.40	49.60	35.40	42.00 ± 4.13
Total	77.50	88.60	90.70	78.50	82.23 ± 4.24

[a] A = androstanedione; P = pregnanedione; A_4 = androstenedione; P_4 = progesterone
[b] 21 hour incubation
[c] $\bar{x} \pm$S.E. does not include endometrium 2(19)

Table 2-6. Percent distribution of radioactivity eluted with standard steroid peaks after CrO_3 oxidation and gas chromatography of conceptus metabolites

	Conceptus (Day)							
Metabolite[a]	1(19)	2(19)	3(19)	4(19)[b]	5(21)	6(22)	7(23)	$\bar{X} \pm$ S.E.[c]
5β-A	3.3	2.8	3.5	8.3	3.1	3.0	1.3	2.83 ± 0.32
5α-A	5.9	3.8	6.6	12.5	2.3	6.0	2.5	4.52 ± 0.77
5β-P	59.9	67.4	42.1	32.4	27.6	15.2	14.9	37.85 ± 9.17
5α-P	9.9	5.5	3.8	2.6	7.7	16.1	12.3	9.22 ± 1.85
A_4	1.3	1.6	3.1	1.8	5.1	7.4	12.5	5.17 ± 1.74
P_4	4.1	5.7	3.7	12.6	13.0	12.2	15.5	9.03 ± 2.09
Total	84.4	86.8	65.9	70.2	58.8	59.9	59.0	69.13 ± 5.32

a A = androstanedione; P = pregnanedione; A_4 = androstenedione; P_4 = progesterone
b 21 hour incubation
c Mean±S.E. does not include Conceptus 4(19)

Figure 2-5. Elution of endometrial steroid metabolites on gas liquid chromatography following CrO_3 oxidation.

PERCENT RADIOACTIVITY ELUTED WITH STANDARD

STEROIDS AFTER GAS/LIQUID CHROMATOGRAPHY OF ENDOMETRIAL METABOLITES

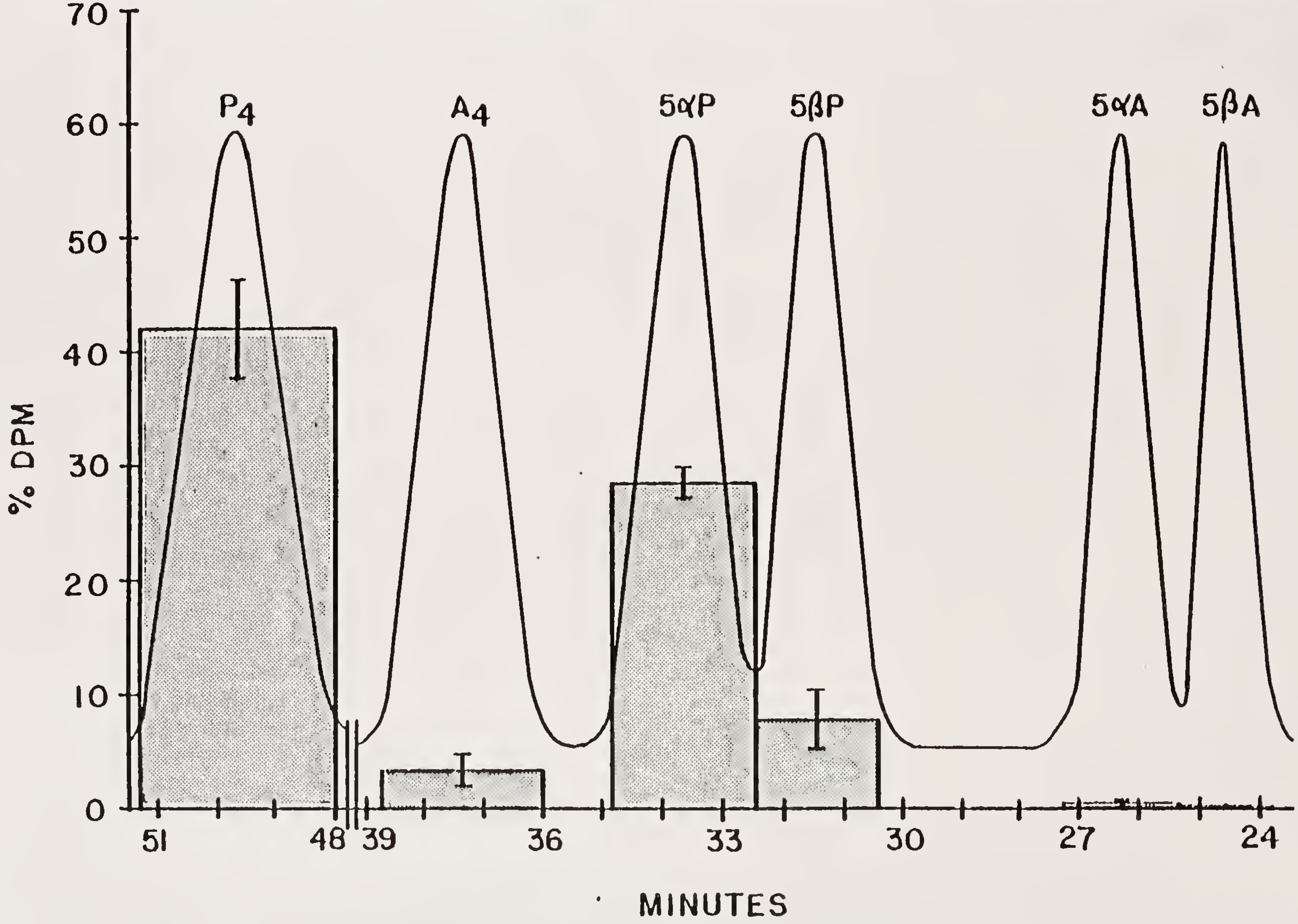
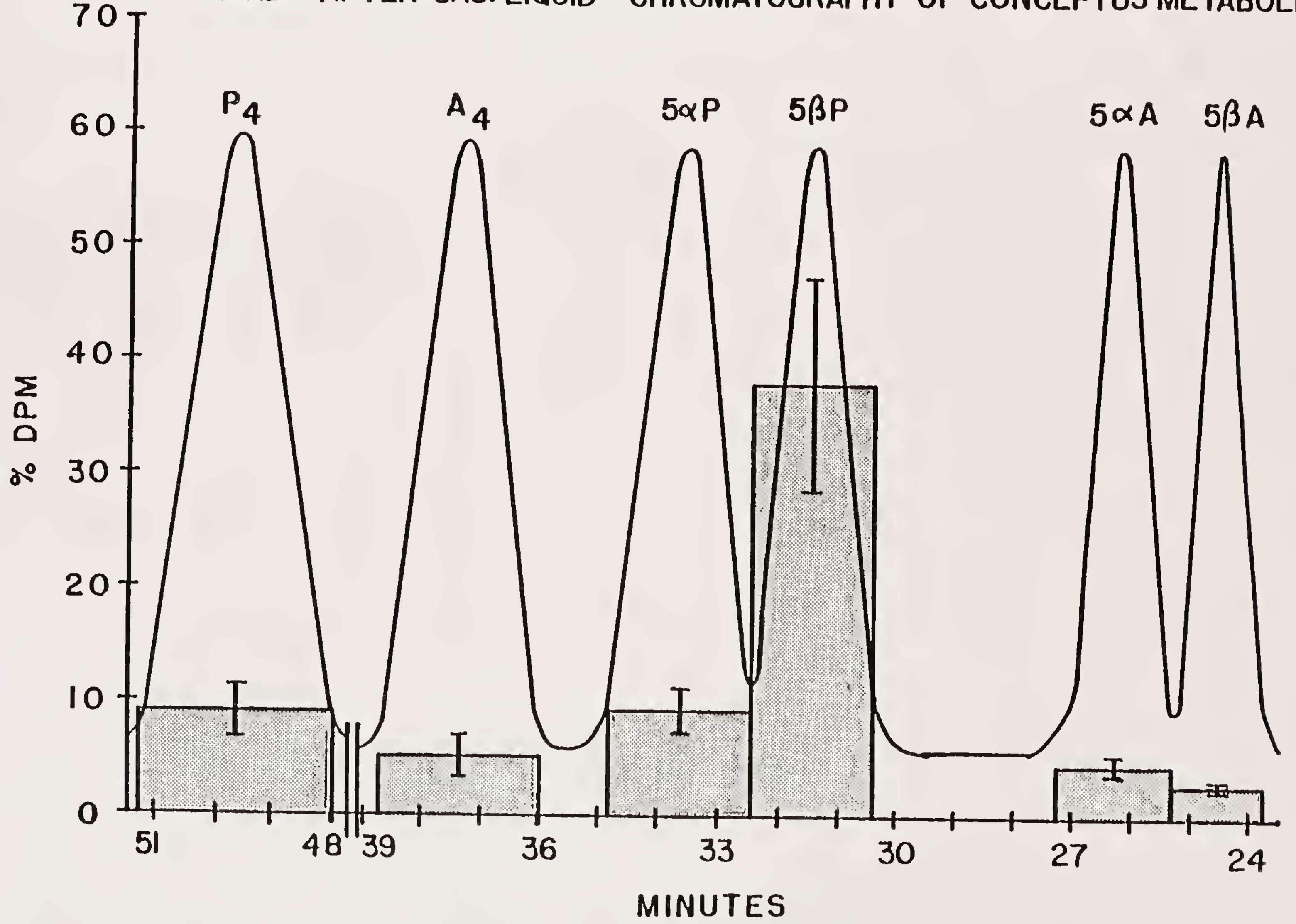


Figure 2-6. Elution of conceptus steroid metabolites on gas liquid chromatography following CrO_3 oxidation.

PERCENT RADIOACTIVITY ELUTED WITH STANDARD STEROIDS AFTER GAS/LIQUID CHROMATOGRAPHY OF CONCEPTUS METABOLITES



of P_4 by conceptus tissues resulted in formation of predominantly 5β-pregnane derivatives. Lesser amounts of radioactivity eluted with 5α-pregnanedione, A_4 and 5α/5β-androstanedione. Although sample numbers are too small to make any firm conclusions, it was interesting to note that percent of 5β-pregnane derivatives appeared to decline in conceptus incubations from day 19 to 23 (Table 2-6; 56.5 ± 6.1 versus 19.2 ± 3.4). This trend was associated with a slight increase in 5α-pregnane derivatives (6.4 ± 1.5 versus 12.0 ± 2.0).

Limited data were generated from GLC analysis of radiolabelled steroid metabolites in areas 2, 3 and 5 of three day 19 conceptus incubations (Table 2-7). The major percent of radioactivity (38.3) in area 2 (n=1) eluted at 4 minutes, prior to the earliest eluting GLC standard (Figure 2-1; Appendix B). Progesterone and 5β-pregnanedione were also major steroids in this area. Small amounts of A_4 were also detected. Area 2 contained 45% of total eluted radioactivity after steroid separation on Sephadex LH20 columns (Table 2-4). Over 60% of the radioactivity in area 3 (n=3) coeluted with 5β-pregnan-3α-ol-20-one on GLC. Nearly a third of radioactivity in area 5 (n=2) coeluted with the GLC standard, 5β-pregnanediol. Although $[^{14}C]$-T markers eluted in area 5 on LH20 column chromatography, no $[^{3}H]$-T was observed in these samples. These data, although based on limited observations, provide preliminary

Table 2-7. Gas liquid chromatography of major conceptus steroid metabolites from LH20 column chromatographic areas

Area	(N)[a]	GLC Standard	(minutes)	% [^3H]	$\overline{X} \pm$ S.E.
2	(1)	Unknown	(4)	38.3	38.3
		5α-Androstanedione	(22)	2.2	2.2
		5β-Pregnanedione	(26)	16.8	16.8
		5α-Pregnanedione	(28)	4.5	4.5
		Androstenedione	(32)	4.0	4.0
		Progesterone	(42)	17.3	17.3
3	(3)	5β-pregnan-3α-ol-20-one	(14)	55.5,68.2,60	61.2 ± 3.0
		5β-pregnanedione	(26)	3.9,2.3,3.6	3.3 ± 0.4
5	(2)	5β-pregnane-3α,20α-diol	(10)	32.3,26.9	29.6±1.9

[a] Individual day 19 bovine conceptuses

indications as to the identity of major steroids synthesized by the bovine conceptus relative to their elution and sephadex LH20 columns (CyH:Bz:MeOH [90:25:5]).

HPLC of Conceptus and Endometrial Metabolites

Procedures followed during identification of conceptus (n=15) and endometrial (n=10) steroid metabolites on HPLC were similar to those used for GLC analyses. Radioinert steroid standards were used as markers to evaluate base structures of oxidized radioactive metabolites. Retention times for radioinert and [^{14}C]-steroid markers used in HPLC analyses are listed in Table 2-8. Nearly 40 percent of total eluted radioactivity from oxidized endometrial incubations were collated with P_4 standards (Table 2-9). The major oxidized metabolite was 5α-pregnanedione (28.8 ± 2.9%). No major differences were observed between days 19 (n=4) and 21 to 23 (n=6) endometrial metabolic patterns, although percent radioactivity eluted with 5α-pregnanedione declined slightly, but increased in 5β-pregnandione fractions as gestational age increased. Little radioactivity coeluted with P_4 standards in oxidized conceptus samples (table 2-10), attesting to the high metabolic activity of conceptus tissues. Approximately 45 percent of radioactivity eluted with 5β-pregnanedione standards. Days 19 (n=9) and 21 to 23 (n=6) oxidized conceptus metabolite patterns differed in percent of radioactivity accumulated in 5β-pregnanedione and 5α-androstanedione fractions. As

Table 2-8. Retention times of steroid markers on HPLC

SYSTEM A[a]

Radioinert Steroids	N	Mean ± S.E. Retention (min)
Testosterone	45	2.01±.003
Androstenedione	44	2.39±.005
5β-Androstanedione	43	2.88±.005
5α-Androstanedione	41	3.46±.009
Progesterone	46	4.88±.010
5β-Pregnan-3α-ol-20-one	45	5.89±.013
5β-Pregnanedione	45	7.08±.016
5α-Pregnanedione	45	7.90±.022
Radioactive Steroids		
[^{14}C]-Androstenedione	37	2.55±.015
[^{3}C]-5β-Pregnane-3α,20α-diol	10	4.70±.015
[^{14}C]-Progesterone	37	5.05±.017

	SYSTEM B[b]		SYSTEM C[c]	
Radioinert Steroids	N	Mean ± S.E. Retention (min)	N	Mean ± S.E. Retention (min)
Estriol	38	2.43±.006	18	2.37±.004
Estradiol-17β	38	4.87±.017	18	8.35±.010
Estrone	38	5.85±.023	18	12.09±.018
Radioactive Steroids				
Estriol	-	-	18	2.56±.016
Estradiol-17β	10	5.00±.035	18	8.56±.014
Estrone	39	6.03±.020	18	12.24±.019

[a] Columns: 5μ guard column (RP-18 OD-GU; Brownlee Labs. Inc., Santa Clara, CA) and 3μ octadecylsilica column (HS-3 C18; Perkin Elmer Inc., Norwalk, CT); solvents: acetonitrile:water (54:46); isocratic, 1.5 ml/minute flow rate.

[b] Columns: 10μ octacecylsilica column (Radial Pack; Waters Associates Inc., Milford, PA); solvents: acetonitrile: water (55:45); isocratic, 1.5 ml/minute flow rate.

[c] Columns: (same as b); solvents: acetonitrile:water (45:55); isocratic, 2 ml/minute flow rate.

Table 2-9. Percent distribution of radioactivity eluted with standard steroids after CrO_3 oxidation and HPLC of endometrial metabolites

Day 19	A_4	5β-A	5α-A	UNK	P_4	5β-P	5α-P
88-1	0	1.0	2.8	8.71	42.73	5.28	31.33
88-2	3.07	1.82	5.0	7.5	31.1	2.56	23.54
6/55-1	1.05	1.02	2.0	4.3	42.55	0	43.47
6/55-2	1.14	1.17	2.68	4.3	38.09	1.76	44.04
Mean	1.32	1.25	3.12	6.2	38.62	2.4	35.6
±S.E.	0.55	0.17	0.56	0.97	2.36	0.95	4.3

Days 21-23	A_4	5β-A	5α-A	UNK	P_4	5β-P	5α-P
8/131-1	1.47	0.47	2.66	3.3	48.02	9.32	33.6
96-2	3.4	2.4	6.11	7.43	15.68	8.32	28.2
8/24-1	0	0	2.6	5.15	61.08	6.01	15.04
8/24-2	0	0	5.1	4.8	46.16	11.99	27.05
75-4	2.72	1.79	4.21	9.8	33.31	7.13	20.1
75-5	2.22	2.22	5.78	8.0	28.7	6.34	21.3
Mean	1.64	1.15	4.41	6.41	38.83	8.19	24.22
±S.E.	0.53	0.42	0.57	0.90	6.01	0.83	2.48
Overall Mean	1.5	1.2	3.9	6.3	38.7	5.9	28.8
±S.E.	±.4	±.3	±.5	±.7	±3.7	±1.1	±2.9

Abbreviations: A_4 (androstenedione), 5β-A (5β-androstanedione), 5α-A(5α-androstanedione), UNK (unknown), P_4 (progesterone), 5β-P (5β-pregnanedione), 5α-P (5α-pregnanedione).

Table 2-10. Percent distribution of radioactivity eluted with standard steroids after CrO_3 oxidation and HPLC of conceptus metabolites

Day 19	A_4	5β-A	5α-A	P_4	3α-OH	5β-P	5α-P
88	4.51	2.8	11.07	1.59	9.67	40.91	4.17
6/55	2.75	2.59	13.2	1.0	12.95	47.31	1.9
71	4.13	2.98	10.29	2.07	8.03	39.2	2.19
8/24	1.74	2.37	3.07	1.91	5.04	65.26	4.35
8/34	2.33	2.36	10.31	2.55	3.87	55.95	5.18
6/95	4.65	2.91	6.99	3.11	6.11	48.54	5.03
5/195	3.43	5.14	5.61	4.6	7.8	52.46	7.07
1/80	3.41	3.42	5.13	2.97	7.92	51.05	4.26
9/96	2.91	2.49	7.96	1.81	11.13	45.32	6.28
Mean	3.32	3.01	8.18	2.40	8.09	49.56	4.49
±S.E.	0.31	0.27	1.03	0.33	0.91	2.49	0.53
Days 21-23	A_4	5β-A	5α-A	P_4	3α-OH	5β-P	5α-P
8/173	5.1	6.69	30.49	2.77	5.38	25.6	2.07
8/131	1.19	3.69	21.14	2.12	9.23	41.96	1.0
8/24	1.61	2.81	13.65	2.55	8.46	43.0	2.05
96	3.75	8.06	14.29	2.98	9.13	40.46	3.71
75	3.94	5.94	11.56	2.07	10.38	40.84	4.01
5/195	1.0	5.1	27.25	1.43	8.22	35.97	1.9
Mean	2.77	5.38	19.73	2.32	8.47	38.0	2.46
±S.E.	0.64	0.72	2.92	0.21	0.63	2.43	0.43
Overall Mean	3.10	3.96	12.80	2.37	8.22	44.92	3.68
±S.E.	±0.32	±0.45	±1.97	±0.22	±0.60	±2.31	±0.44

Abbreviations: A_4 (androstenedione), 5β-A (5β-androstanedione), 5α-A(5α-androstanedione), P_4 (progesterone), 3α-OH (5β-pregnan-3α-ol-20-one), 5β-P (5β-pregnanedione), 5α-P (5α-pregnanedione).

observed in GLC analyses, 5β-pregnanedione associated radioactivity decreased (~10%) as gestational age of the conceptus increased (Table 2-10). Conversely, percent radioactivity eluting with 5α-androstanedione on HPLC increased by approximately 10 percent in days 21 to 23 versus day 19 conceptus incubations. Incomplete oxidation of 5β-pregnane metabolites was suggested since 8 percent of radioactivity eluted with 5β-pregnan-3α-ol-20-one standards. This result may suggest that a major percent of the 5β-pregnane metabolite pool consisted of 5β-pregnan-3α-ol-20-one (Tables 2-7 and 2-11). High performance liquid chromatographic analyses of metabolites in separate LH20 column chromatographic areas 2, 3 and 5 were performed on pooled days 19 (n=9) and 21 to 23 (n=6) conceptus incubations (Table 2-11). Results are similar to data generated on GLC (Table 2-7). Conceptus area 2 was composed largely of P_4, 5β-pregnanedione and 5β-pregnan-3α-ol-20-one. Radioactivity eluted with 5β-pregnan-3α-ol-20-one probably represented residual spillover from conceptus area 3 from LH20 column chromatography. Two regions of radioactivity were not associated with radioinert HPLC standards (unknown 1 and 2). The earlier unknown eluted with the void volume. Elution times of [^{3}H]-5β-pregnane-3α,20α-diol standards, evaluated in separate HPLC runs, were very similar to elution time of the latter unknown. However, it is doubtful that unknown 2 radioactivity in area 2 samples

Table 2-11. HPLC of major conceptus steroid metabolites from LH20 column chromatographic areas; % of total eluted radioactivity

	UNK1	A_4	5β-A	5α-A	UNK2	P_4	3α-OH	5β-P	5α-P
POOLED DAY 19 CONCEPTUSES									
AREA 2 (n=9)	11.8	3.0	2.5	6.0	15.8	18.7	17.1	20.0	5.2
AREA 3 (n=9)	11.0	1.2	1.7	5.2	4.0	7.9	61.5	5.8	0
AREA 5 (n=7)	34.5	0	5.3	5.9	8.21	0	34.3	3.7	0
OXIDIZED AREA 5 (n=7)	4.2	0	6.1	7.6	0	0	11.4	64.3	0
POOLED DAYS 21-23 CONCEPTUSES									
AREA 2 (n=6)	13.3	3.6	2.8	10.7	13.3	22.0	14.6	15.1	4.5
AREA 3 (n=6)	12.0	0	2.8	6.1	7.6	6.7	55.6	6.1	0
AREA 5 (n=6)	19.1	0	3.1	5.3	11.3	0	50.7	5.4	0
OXIDIZED AREA 5 (n=6)	0	0	2.5	4.9	0	0	15.5	69.9	0

Abbreviations: UNK-1 (early eluting unknown), A_4 (androstenedione), 5β-A (5β-androstanedione), 5α-A(5α-androstanedione), UNK-2 (late eluting unknown), P_4 (progesterone), 3α-OH (5β-pregnan-3α-ol-20-one), 5β-P (5β-pregnanedione), 5α-P (5α-pregnanedione).

represent 5β-pregnane-3α,20α-diol since this standard elutes in area 6 and 7 when run on LH20 columns (CyH:Bz:MeOH [90:25:5]). Small percentages of A_4 and 5β/5α-reduced androgens were also present in pooled area 2 samples. The predominant (approximately 60%) radioactive conceptus metabolite in area 3 coeluted with the 5β-pregnan-3α-ol-20-one standard on HPLC. This is in agreement with GLC data described earlier (Table 2-7). Pooled area 5 radioactivity eluted largely with 5β-pregnan-3α-ol-20-one standards and in unknown regions 1 and 2. Radioactivity in unknown 1 eluted in a broad region from 0.6 to 2.2 minutes on HPLC. A portion of unknown 1 radioactivity eluted in the region of radioinert T standards. Oxidation of the 17β-hydroxyl group on T would yield A_4 (Bush, 1961). Therefore, area 5 was oxidized to evaluate possible T production by conceptus tissues. No radioactivity eluted with A_4 standards following oxidation suggesting that T was not a component of radioactivity in area 5 (unknown 1). Additionally, radioactivity in unknown regions 1 and 2 appeared to be converted to 5β-pregnanedione following CrO_3 oxidation. No changes in percent radioactivity eluting with 5α/5β-reduced androgens was observed. Thus, radioactive metabolites in unknown regions 1 and 2 appeared to be composed of hydroxylated 5β-pregnane metabolites.

Conceptus Estrogen Production (HPLC Analyses)

Conceptus (n=15) and endometrial (n=10) estrogen production was evaluated in pooled estrogen fractions, viz., from [^{14}C]-E_1 elution to approximately 120 ml, collected following LH20 column chromatography. Retention times for radioinert and [^{14}C]-estrogen markers used in HPLC analyses are listed in table 2-8). No estrogens were identified from endometrial incubations, however a major peak of radioactivity (48.7 ± 5.2%) eluted between E_3 and E_2 standards. A similar, although smaller, peak of radioactivity eluted between E_3 and E_2 standards in conceptus incubations (Figure 2-7).

Definitive peaks of [^{3}H]-metabolites coeluted with [^{14}C]- and radioinert E_1, E_2 and E_3 markers in all conceptus incubations (Table 2-12; Figure 2-7). Estradiol-17β appears to be the primary estrogen synthesized from P_4 substrate with lesser amounts of E_1 and E_3 being produced. Mean ± S.E. of total estrogen production was 44.12 ± 5.47 pg/conceptus incubation (Appendix C). No differences in conceptus estrogen production were noted between days 19 and 21 to 23 of gestation.

Discussion

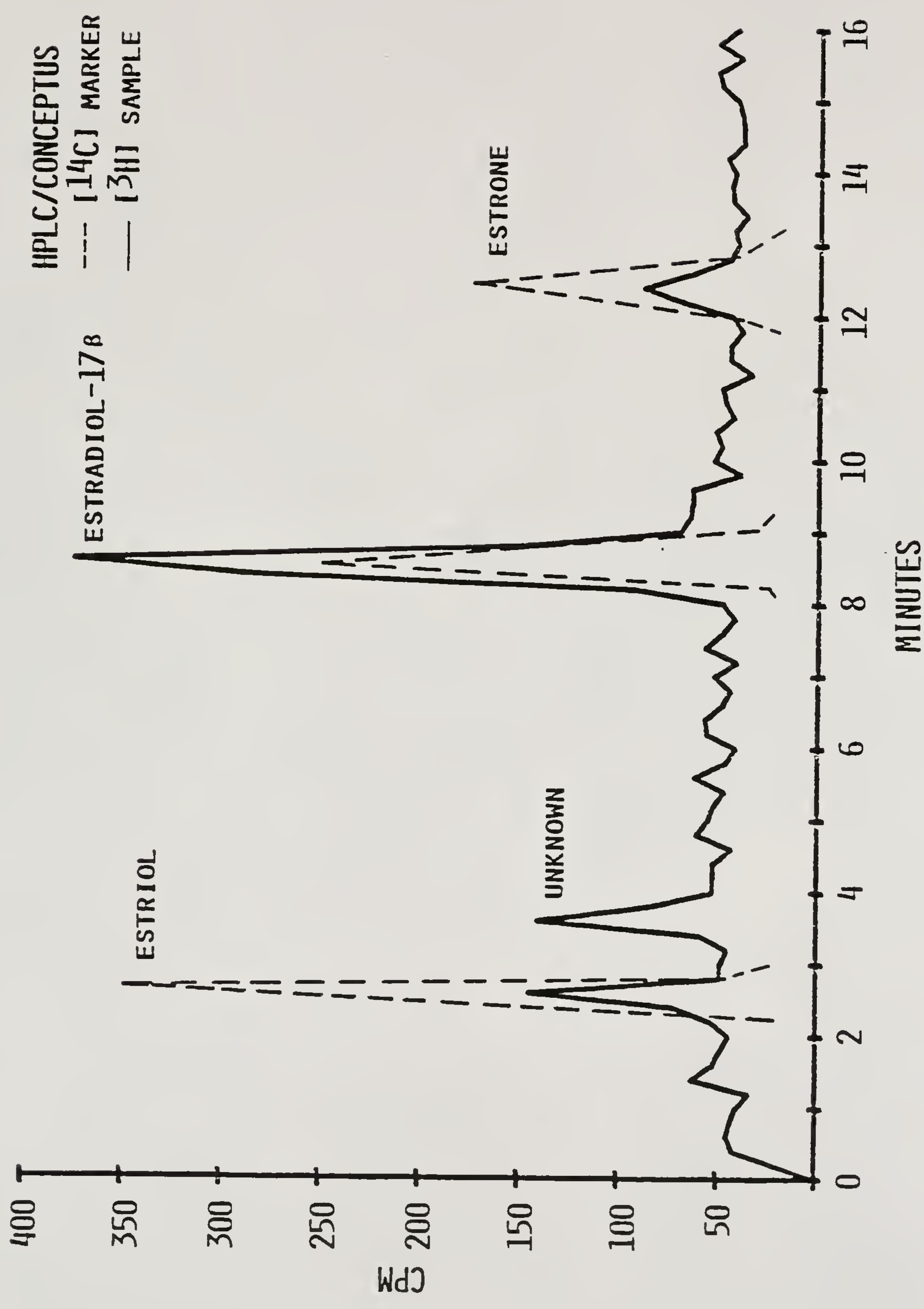
Results of the present study support previous data which indicated a differential metabolism of neutral steroids (P_4 and A_4) by bovine conceptus and endometrial

Table 2-12. Bovine conceptus estrogen production; HPLC determinations

Conceptus No.	Wet wt. (mg)	Day	E_3 (pg total)[a]	E_2 (pg total)	E_1 (pg total)	Total Estrogens (pg)
88	312	19	1.54	5.83	1.02	8.39
9/96	207.6	19	12.0	9.83	9.13	30.96
71	284	19	N.D.	12.14	N.D.	12.14
6/55	291.3	19	1.53	23.30	2.27	27.1
5/195	140	19	1.28	44.52	1.94	47.74
8/24	43	19	2.61	54.32	3.46	60.39
8/34	50	19	1.97	73.20	2.85	78.02
6/95	159	19	2.06	65.65	2.31	70.02
1/80	255	19	3.11	88.80	2.29	94.2
Mean	193.54		2.9	41.95	2.81	47.66
±S.E.	32.02		1.1	9.6	0.81	9.5
75	53	21	3.72	28.89	1.88	34.49
8/173	364.7	22	1.07	34.83	2.19	38.09
96	336	23	5.83	13.00	22.25	41.08
8/24	356.2	23	3.39	31.74	4.83	39.96
5/195	370.6	23	1.15	32.11	4.73	37.99
8/131	360.5	23	1.10	35.84	4.35	41.29
Mean	306.8		2.71	29.40	6.70	38.81
±S.E.	46.56		.73	3.13	2.88	0.95
Overall Mean	238.83		2.82	36.93	4.37	44.12
±S.E.	30.35		.73	6.10	1.34	5.47

[a] See Appendix C for mass calculations

Figure 2-7. Sample elution pattern of conceptus (day 23) estrogens analyzed by high performance liquid chromatography (acetonitrile:water, 45:55).


HPLC/CONCEPTUS
--- [14C] MARKER
— [3H] SAMPLE
ESTRIOL
UNKNOWN
ESTRADIOL-17β
ESTRONE
CPM
400
350
300
250
200
150
100
50
0
2
4
6
8
10
12
14
16
MINUTES

tissues, viz., conceptus 5β-reductase activity versus endometrial 5α-reductase activity (Chenault, 1980; Eley et al., 1983). In addition, GLC and HPLC analyses of conceptus and endometrial P_4 metabolism demonstrated production of small quantities of A_4 and 5α/5β reduced androgens. Conceptus and endometrial incubations also contained small quantities of 5α and 5β-reduced progestins, respectively. This apparent 5α/5β reduction of P_4 by conceptus and endometrial tissues, respectively, may represent endogenous reductase enzyme activities or result from cross-contamination of conceptus and endometrial tissues or cells. Endometrial 5β-reductase activity, as determined by GLC and HPLC analyses of oxidized samples, appeared to increase slightly after day 19 of gestation. During this period, conceptus-derived binucleated giant cells are known to migrate and fuse with endometrial epithelial cells such that by day 24 of gestation multinucleated giant cells were reported to comprise 25% of the total endometrium epithelial cell population and 50% of cell area in the gravid uterine horn (Wathes and Wooding, 1980). Based on this evidence, it may be suggested that endometrial 5β-reductase activity is a result of conceptus cell migration into this tissue. Reduction of P_4 to 5α-androstane and pregnane derivatives appeared greatest in later stage conceptuses in the present study. This was most evident when total 5α based steroids were evaluated in oxidized samples. Our results conflict in

this respect with observations of Chenault (1980) and Eley et al. (1983), however, these authors evaluated 5α/5β reduction in specific chromatographic fractions as opposed to total oxidized conceptus pools in the present study. Increased 5α-reductase activity may reflect conceptus developmental changes, viz., embryo organogenesis, allantois expansion, yolk sac regression, vascularization of the chorioallantois (Greenstein and Foley, 1958a,b; Greenstein et al., 1958). Endometrial cell contamination of conceptus incubations seems doubtful since firm attachment between these tissues does not occur until day 27 (King et al., 1980). However, this possibility cannot be excluded.

Identification of 5β-pregnan-3α-ol-20-one as a major P_4 metabolite in conceptus incubations agrees with findings of Eley et al. (1983). Over 40 percent of radioactivity following conceptus incubations with $[^3H]$-P_4 eluted in a single sharp peak comprising area 3 on LH20 column chromatography. Sixty percent of radioactivity in this peak coeluted with 5β-pregnan-3α-ol-20-one on GLC and HPLC. Thus, conceptus tissues produced nearly 5 ng of this steroid during 3 hour incubations with $[^3H]$-P_4 (20 ng). In addition, day 21 to 23 conceptus incubations metabolized P_4 to more polar compounds eluting as a double peak in area 6 (30 percent of total radioactivity). Unfortunately, metabolites in this area were not evaluated in the present study. However, Eley et al. (1983) identified

5β-pregnane-3α,20α-diol as a major conceptus metabolite of P_4. It is possible that area 6 radioactivity represents dihydroxylated pregnane and androstane derivatives in the present study. Production of more polar compounds as conceptus age increases may be due to increased tissue mass and tissue activity, as suggested by data in the present study and Eley et al. (1983). Based on proportions of 5β-pregnanedione, 5β-pregnan-3α-ol-20-one and 5β-pregnane-3α,20α-diol identified in the present study and by Eley et al. (1983), it may be suggested that sequential P_4 metabolism occurs by 5β-reduction (5β-reductase) and rapid 3α-hydroxylation (3α-hydroxysteroid dehydrogenase; HSD), followed by 20α hydroxylation (20α-HSD) in later stage conceptuses.

The present report consistently demonstrated production of E_1, E_2 and E_3 following bovine conceptus tissue incubations with P_4. The ability of bovine conceptus tissues to utilize C21 steroids as precursors for estrogen biosynthesis was suggested by Shemesh et al. (1979). However, Eley et al. (1983) was unable to demonstrate estrogen production using [^{3}H]-P as substrate. Sensitivity of HPLC techniques employed to isolate estrogens in the present study may be superior to methods used by Eley et al. (1983), viz., column chromatography, phenolic extraction, acetylation and recrystallization, thus explaining the apparent discrepancies in our two reports. Extradiol-17β

was the primary estrogen identified from days 19 to 23 conceptus incubations in the present study. This is interesting in light of observations during later gestation in cattle when E_1 production predominates (Eley et al., 1979a; Robertson and King, 1979).

Functions of conceptus 5β-reduced steroids and estrogens during early pregnancy in cattle are not known. However, Chenault (1980) and Eley et al. (1983) have cited evidence suggesting that these steroids may be involved in conceptus development processes and alterations in maternal uterine physiology. For example, 5β-reduced steroids have been shown to stimulate erythropoiesis (Gordon et al., 1970; Grass and Goldwasser, 1972; Singer and Adamson, 1976), hemoglobin biosynthesis (Necheles and Rai, 1969; Levere et al., 1967), and activity of δ-aminolevulinic acid synthetase, the rate limiting enzyme of porphyrin-heme biosynthesis (Edwards and Elliott, 1975). Thus, 5β-reduced steroids may play a role in development of the conceptus circulatory system during early pregnancy (see: Greenstein and Foley, 1958a,b; Greenstein et al., 1958; Grimes et al., 1958). Kubli-Garfias et al. (1979) demonstrated that 5β-reduced progestins were several times more potent than P_4 in reducing uterine myometrial contractions, whereas 5α-reduced progestins were less effective than P_4 in this regard. These 5β-steroids may decrease myometrial contractility via regulation of myometrial Ca^{+2} availability during early

pregnancy (Crankshaw et al., 1979). Human mammary tumor 5β-reductase activity was negatively correlated with estrogen receptor concentrations in that tissue (Abul Hajj, 1979). In addition, Findlay et al. (1982) reported that decreased caruncular E_2 receptor concentrations in ovine uteri were associated with location of conceptus tissues within the uterus. Estrogens stimulate uterine $PGF_{2\alpha}$ production (Chapter 3) and initiate luteolysis (Eley et al., 1979b) in cattle. This process may be prevented during early pregnancy by conceptus 5β-steroid effects on endometrial E_2 receptor populations. Conversely, 5β-reductase activity by conceptus tissue may be a means of eliminating excessive aromatizable substrate or otherwise reducing quantities of active steroids within the uterine lumen.

Initiation of bovine conceptus estrogen biosynthesis (Shemesh et al., 1979; Chenault, 1980; Gadsby et al., 1980; Eley et al., 1983) coincides with transient elevations in uterine blood flow during early pregnancy. Furthermore, exogenous estrogens are known to stimulate uterine blood flow in cyclic cattle (Roman-Ponce et al., 1979; Chapter 3). Catecholestrogens, hydroxylated metabolites of estrogen, were suggested to mediate the E_2-induced uterine blood flow response (Ford, 1978, 1982; Ford and Reynolds, 1983). These estrogen metabolites also induce production of P_4 receptors in the rat uterus (Kirchhoff et al., 1983). Thus, conceptus derived estrogens and estrogen metabolites

may be involved in maintaining an embryotropic uterine environment during early gestation in cattle.

In conclusion, data presented provide some insight as to the patterns and types of P_4 metabolites produced by bovine conceptus and endometrial tissues between days 19 and 23 of gestation. Evidence supports conceptus production of androgens and estrogens from P_4 substrate with E_2 comprising approximately 80% of total estrogen biosynthesis by the bovine conceptus. Differential-5α/5β reduction of neutral steroids by endometrial and conceptus tissues, respectively, and function of 5β-reduced conceptus steroids during early gestation warrant evaluation in the future.

CHAPTER 3
UTERINE PROSTAGLANDIN AND BLOOD FLOW RESPONSES TO ESTRADIOL-17β IN CYCLIC CATTLE

Introduction

Free and conjugated estrogens shorten lifespan of the corpus luteum (CL) in cattle if administered during the luteal phase of the estrous cycle (Greenstein et al., 1958; Wiltbank, 1966; Eley et al., 1979). Luteolytic activity of estrogen is thought to be mediated through stimulation of uterine prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthesis and release. The luteolytic activity of exogenous $PGF_{2\alpha}$ in cattle has been documented extensively (Hansel et al., 1973; Hafs et al., 1974; Lauderdale, 1974; Thatcher and Chenault, 1976).

Elevated levels of $PGF_{2\alpha}$ in uterine venous drainage (Nancarrow et al., 1973; Shemesh and Hansel, 1974), uterine tissue (Shemesh and Hansel, 1975) and uterine flushings (Lamothe et al., 1977; Bartol et al., 1981a) coincide with the transient rise in follicular estradiol prior to ovulation in cyclic cattle (Nancarrow et al., 1973; Chenault et al., 1975). Collectively, these observations support the concept that follicular estrogen secretion regulates onset of luteolysis.

Peterson et al. (1975) and Kindahl et al. (1976) reported elevations in peripheral plasma 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM), the inactive metabolite of $PGF_{2\alpha}$, which were temporally associated with CL regression in cyclic heifers. Thatcher and coworkers (1979) also utilized measurements of PGFM in the peripheral circulation to characterize indirectly the uterine response to exogenous estradiol in cyclic heifers. Concentrations of PGFM began to rise by 3 h, reached peak levels by 6 h, and returned to basal values by 9 h post-estradiol administration. In addition, estrous cycle length was reduced in estradiol-treated heifers as compared with heifers treated with vehicle alone. An additional study indicated that this increase in peripheral plasma PGFM concentration was associated with an elevation of $PGF_{2\alpha}$ in uterine flushings collected at 6 h after estradiol injection (Bartol et al., 1981b). Such a response further supports the use of PGFM measurement in peripheral circulation as an index of uterine $PGF_{2\alpha}$ production.

In cyclic cattle, luteolysis is thought to occur via a local, countercurrent exchange of $PGF_{2\alpha}$ from the ovarian vein, draining the uterine horn ipsilateral to the ovary bearing the CL, into the adjacent ovarian artery (see reviews: Baird, 1978; Ginther, 1981). Hixon and Hansel (1974) presented evidence supporting such an exchange of $PGF_{2\alpha}$ from the uterine venous drainage into the ovarian

arterial supply following intrauterine deposition of $PGF_{2\alpha}$ in cyclic cattle. In a subsequent experiment (Shemesh and Hansel, 1975), a countercurrent exchange of $PGF_{2\alpha}$ was not detected. However, only single samples were collected at surgery and a more chronically sustained sampling regime is warranted to test the countercurrent exchange concept.

Objectives of this experiment were 1) to examine directly the bovine uterine response to exogenous estradiol-17β (E_2) by characterizing uterine venous concentrations of $PGF_{2\alpha}$ and PGFM, uterine blood flow (UBF) and uterine production of $PGF_{2\alpha}$ and PGFM; 2) to describe the temporal relationship between peripheral plasma PGFM concentrations and uterine production of $PGF_{2\alpha}$ and PGFM following E_2 administration; and 3) to examine the possible countercurrent exchange of $PGF_{2\alpha}$ from uterine venous drainage into the ovarian arterial supply during the period of E_2-induced $PGF_{2\alpha}$ production by the uterus.

Materials and Methods

Normal cyclic dairy cows (n=7) were used to characterize uterine responses to exogenous E_2 injection. Animals were prepared for surgery on day 17 of the estrous cycle (day of estrus = day 0). Five grams of sodium thiamylal (BIO-TAL, Bio-Ceutic Laboratories, Inc., St. Joseph, MO) were dissolved in 40 ml of sterile saline and injected via

the jugular vein. An endotracheal tube was fitted and cows maintained under general anesthesia with a fluothane (Vescor, Orlando, FL) and oxygen gas mixture. The surgical field was shaved, disinfected and covered with a sterile drape. The reproductive tract was exposed following a mid-ventral laparotomy and specific vessels (Ginther, 1976) ipsilateral to the CL were catheterized: uterine branch of the ovarian vein (with the catheter tip located 15 to 20 cm into the main ovarian vein (OV, n=7) and uterine branch of the ovarian artery (UBOA, n=5). Ideally, blood from the uterine branch of the ovarian vein should be used for measuring uterine venous prostaglandin concentrations. However, maintaining patency of the catheter in this vessel was difficult. Since little ovarian prostaglandin contribution was expected, the larger ovarian vein was utilized. Catheters were secured within the vessels by ligation at the point of catheter entry into the vessel. This prevented blood flow past the point of catheter entry. An electromagnetic blood flow transducer was placed and secured around the main uterine artery (UA, n=3) or its primary branch (n=2) both ipsilateral to the CL, and a catheter placed in the facial artery (FA, catheter tip inserted into the internal carotid artery, n=7). Blood flow transducers were calibrated prior to surgery within a flow range of 0 to 300 ml/min using excised uterine arteries and heparinized bovine blood (Roman-Ponce et al., 1978). A Narcomatic, Model

RT-500, blood flow meter was used with a Narco Desk Model DMP-4B physiograph (Narco Biosystems, Inc., Houston, TX) to monitor UBF continuously during the experiment. Ampicillin (POLYFLEX, Bristol Laboratory, Syracus, NY) was administered intra-abdominally (375 mg) and intramuscularly (875 mg) prior to closure of the incision.

On day 18, blood samples were drawn concurrently from the three catheters every 30 min, UBF monitored continuously from 1 h pre- to 12 h post-E_2 administration (3 mg in 6 ml of an ETOH:0.9% NaCl [50:50] solution via jugular vein). Blood samples were drawn into heparinized syringes and placed immediately on ice until centrifugation at 12,000 x g (within 15 min of collection). Plasma was stored frozen at -20 C until assayed for $PGF_{2\alpha}$ and PGFM. Blood flow transducers were recovered following conclusion of the experiment via flank laparotomy. Both placement and calibration of the transducers were reconfirmed. One month following the experiment, animals were slaughtered and reproductive tracts dissected to verify placement of uterine catheters.

Unextracted plasma samples (.05, .1 and/or .2 ml) were assayed for PGFM and $PGF_{2\alpha}$. A polyethylene glycol radioimmunoassay system for PGFM was performed in accordance to procedures described by Guilbault et al. (1984). Inter- and intraassay coefficients of variation for a reference sample (100 pg/.2 ml) assayed in duplicate for 10 assays (103.4 $\pm$ 2.9 pg/.2 ml) were 17.0% and 12.5%, respectively.

Prostaglandin $F_{2\alpha}$ was measured using an antibody (gift from Dr. K. Kirton of the Upjohn Company, Kalamazoo, MI) generated in goats against $PGF_{2\alpha}$ conjugated to bovine serum albumin at the C^1-position. Tritiated $PGF_{2\alpha}$ ([5,6,8,9,11,12,14,15-^{3}H(N)], specific activity 150 to 180 Ci/mmole), was purchased from New England Nuclear (Boston, MA). Cross-reactivities of the $PGF_{2\alpha}$ antiserum with other prostaglandins were 53.6% for $PGF_{1\alpha}$; .22% for 15-keto-$PGF_{2\alpha}$; <.04% for PGFM, PGE_1, PGE_2 and arachidonic acid. Standard curves were prepared by adding known amounts of radioinert $PGF_{2\alpha}$ (0, 10, 25, 50, 100, 250, 500, 1000 and 2500 pg) to bovine plasma (.2 ml) obtained from cattle treated with flunixon meglamine (BANAMINE, Schering Corp., Kenilworth, NJ), an inhibitor of cyclo-oxygenase, to suppress prostaglandin synthesis. Cattle were injected twice with Banamine (20 ml each; 50 mg/ml) at 1700 h and 0800 h, and blood collected at 1100 h. Immunoreactive $PGF_{2\alpha}$ in this plasma was below detectable levels (10 pg). Using an antibody dilution of 1:2000, sensitivity of the assay was 10 pg. Unextracted plasma samples were assayed for $PGF_{2\alpha}$ in duplicate using aliquots of either 50, 100 or 200 µl. No significant differences were found between like concentrations of $PGF_{2\alpha}$ measured using these aliquot volumes ($P>.10$). A plasma sample (from uterine branch of the ovarian vein of a day 2 postpartum cow) that contained approximately 8000 pg/ml of immunoreactive $PGF_{2\alpha}$ was assayed

serially in sample volumes of 10, 25, 50, and 100 µl. Samples were brought to a 200 µl assay volume with addition of plasma from Banamine treated cattle. A quantitative linear displacement curve was achieved ($\hat{Y} = 2.43 - 2.052X$; $\hat{Y}$ = logit or natural log of bound/free, X = $\log_{10}$ of sample volumes). Test for homogeneity of regression between this curve and the standard curve ($\hat{Y} = 4.9590 - 2.2967X$) did not show them to differ ($P>.10$; i.e., there was no evidence for lack of parallelism between the standard curve and the displacement curve of bovine plasma). Accuracy of the assay procedure was further characterized by measuring known quantities of exogenous $PGF_{2\alpha}$ added to 200 µl of plasma (from cattle treated with Banamine) at concentrations of 25, 50, 100, 125, 200, 250 and 500 pg. Differences among concentrations were described by a linear regression of added vs. measured $PGF_{2\alpha}$ [$\hat{Y} = -8.7 + 1.01X$; $\hat{Y}$ = amount $PGF_{2\alpha}$ measured (pg/200 µl), X = amount $PGF_{2\alpha}$ added (pg/200 µl), $R^2 = .97$]. The intraassay coefficient of variation for the validation procedure was 14.8%. Inter- and intraassay coefficients of variation for 12 assays were 8.1% and 12.9%, respectively, when duplicate estimates were run in the assay for a 500 pg/.2 ml (582.1 ± 13.9) reference sample.

Uterine production of $PGF_{2\alpha}$ or PGFM (µg/min) from the uterine horn ipsilateral to the CL was calculated by subtracting peripheral (FA) prostaglandin concentrations from OV concentrations and multiplying this difference (OV - FA;

pg/ml) by the UBF (ml/min x 10^{-2}). Least squares mean values of blood flow for a 30 min period were calculated from means of 15 consecutive 1 min intervals prior to each 30 min blood sample. Data were analyzed by method of least squares analysis of variance according to General Linear Models Procedures of the Statistical Analysis System (Barr et al., 1979). Sources of variation included in the mathematical models were cow and time to describe the time trends post-injection of estradiol.

Results

Mean concentrations (pg/ml) of $PGF_{2\alpha}$ and PGFM in the OV and PGFM in the FA were elevated (P<.01) following E_2 injection as compared to pre-treatment concentrations. However, mean concentrations of $PGF_{2\alpha}$ in FA for both pre- and post-E_2 periods were basal (Table 3-1). Least squares regression curves (Figure 3-1; Table 3-2) for net concentrations of $PGF_{2\alpha}$, PGFM and total prostaglandin in OV (i.e., OV - FA pg/ml) following E_2 injection indicated an initial rise above baseline concentrations between 2 to 3 h, a peak at 6 h and a decline to near baseline concentrations by 10 h. A secondary rise in net ovarian vein $PGF_{2\alpha}$ and total prostaglandin concentrations occurred between 10.5 and 12 h. In contrast, net PGFM concentrations in the OV continued to decline until 12 h.

Table 3-1. Least squares means ± S.E. for concentrations of $PGF_{2\alpha}$, PGFM (pg/ml) and uterine blood flow (ml/min) during the pre- and post-estradiol-17_{β} injection periods

Response	UBF* (ml/min)	Ovarian Vein		Facial Artery		UBOA+	
		$PGF_{2\alpha}$	PGFM	$PGF_{2\alpha}$	PGFM	$PGF_{2\alpha}$	PGFM
Pre-E_2	77.0 ±1.9	95.7 ±9.3	121.5 ±10.2	86.8 ±5.6	75.8 ±7.7	76.5 ±15.8	132.3 ±2.1
Post-E_2**	179.4 ±4.1	1382.8 ±128.4	955.5 ±63.6	91.0 ±5.5‡	206.9 ±12.7	599.9 ±115.0	685.2 ±110.3

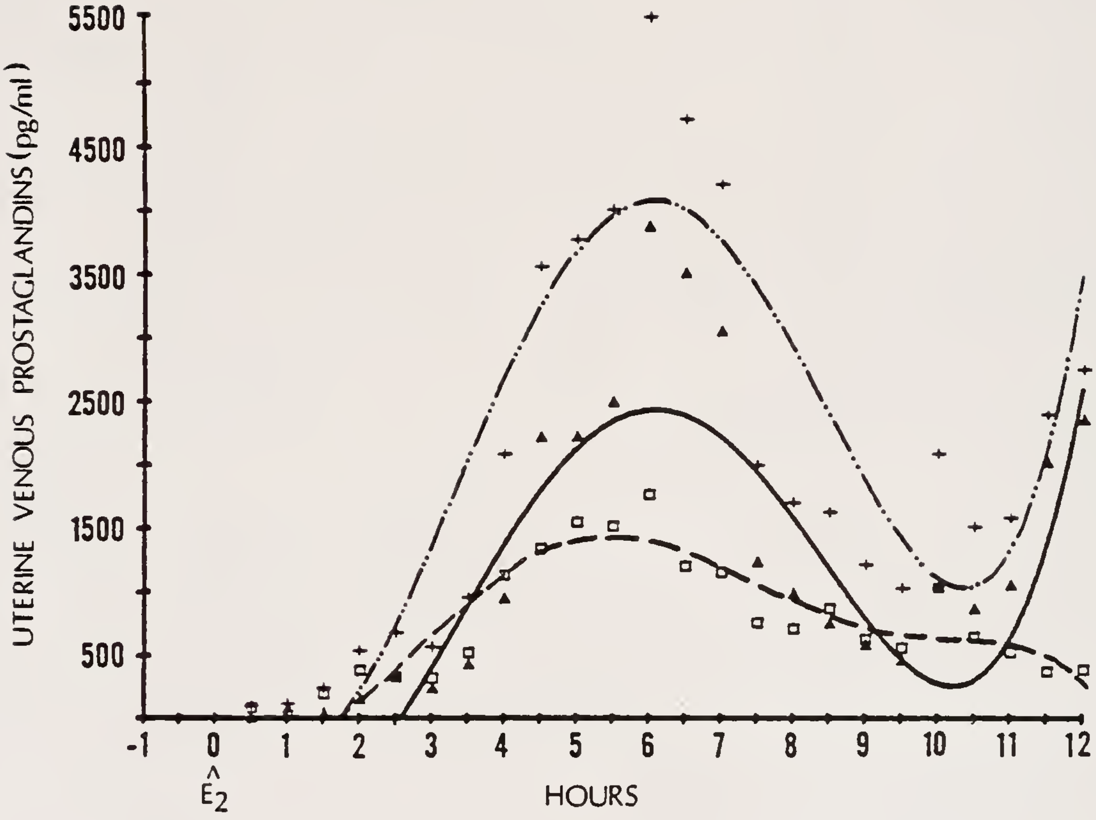
* Uterine Blood Flow

\+ Uterine Branch of the Ovarian Artery

**Least squares means listed in the post-E_2 injection period are greater than the pre-E_2 injection period ($P<.01$)

‡ No significant difference between pre- and post-E_2 concentration ($P>.10$)

Figure 3-1. Least squares means (symbols) and regression lines for ovarian vein (i.e., OV-FA) $PGF_{2\alpha}$ (Δ, ———), PGFM (□ , -----) and total prostaglandin ($PGF_{2\alpha}$+PGFM; +,) concentrations following E_2 injection.



UTERINE VENOUS PROSTAGLANDINS (pg/ml)
5500
4500
3500
2500
1500
500
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
$\hat{E}_2$
HOURS

Table 3-2. Least squares polynomial regression equations for dependent variables

Dependent variables[a]	B_0	B_1	B_2	B_3	B_4	B_5	N	R^2
OV PGFM net pg/ml	6201.3	$-3037.60X$[b]	$514.287X^2$	$-36.6753X^3$	$1.17411X^4$	$-0.013998X^5$	148	.39
OV PGF net pg/ml	7960.9	$-3605.78X$	$502.720X^2$	$-25.6031X^3$	$0.43174X^4$		148	.43
OV TOTAL net pg/ml	9127.6	$-4131.35X$	$598.002X^2$	$-30.8017X^3$	$0.51962X^4$		148	.42
PGFM PRD pg/min	934882	$-465343.0X$	$80231.44X^2$	$-5895.256X^3$	$196.5855X^4$	$-2.45785X^5$	85	.35
PGF PRD pg/min	1226671	$-554791.2X$	$79092.40X^2$	$-4025.961X^3$	$66.9110X^4$		85	.47
TOTAL PRD pg/min	1284674	$-597977.6X$	$88012.32X^2$	$-4501.612X^3$	$74.5083X^4$		85	.43
FA PGFM pg/ml	2197.9	$-1001.41X$	$158.494X^2$	$-10.484X^3$	$0.3035X^4$	$-0.00316X^5$	167	.47
FA PGF pg/ml	83.2	$-1.24X$					167	.52
UBF ml/min	118.8	$-65.14X$[c]	$23.456X^2$	$-2.3930X^3$	$0.09570X^4$	$-0.001331X^5$	116	.69

[a] OV=ovarian vein; FA=facial artery; PGFM=15-keto-13,14-dihydro-PGF; PGF=prostaglandin $F_{2\alpha}$; TOTAL=PGF+PGFM; UBF=uterine blood flow; PRD=net production; net=OV concentration-FA concentration.

[b] X=coded sampling sequence $3<X<27$; coded sample sequence is 30 minutes periods from 0 to 12 h post-E_2 injection.

[c] X=coded sampling sequence $1<X<27$; coded sample sequence is 30 minute periods from -1 h pre- to 12 h post-E_2 injection.

During the 1 h pre-treatment period, UBF was stable and concentrations of $PGF_{2\alpha}$ and PGFM remained low in all vessels (Table 3-1). Fifteen to 30 min following E_2 administration, UBF increased to peak values between 2.5 and 3.5 h (Figure 3-2; Table 3-2) and declined over the period from 4 to 8.5 h. A small secondary rise and fall in UBF occurred between 9 and 12 h.

Profiles of uterine $PGF_{2\alpha}$, PGFM and total prostaglandin production (μg/min; Figure 3-3; Table 3-2) were similar to profiles of net OV concentrations depicted in figure 3-1. Maximal production of uterine $PGF_{2\alpha}$ (.93 ± .25 μg/min), PGFM (.35 ± .08 μg/min), and total prostaglandin (1.26 ± .32 μg/min) were observed at 6 h post-E_2. Facial artery PGFM concentrations were positively correlated (within cow partial correlation) with uterine $PGF_{2\alpha}$ production ($r=.66$, $P<.001$; Figure 3-4) total uterine prostaglandin production ($r=.59$, $P<.001$) and other uterine prostaglandin responses (Table 3-3). Peripheral (FA) $PGF_{2\alpha}$ concentrations were not correlated ($r=.05$, $P>.10$) with any measured uterine prostaglandin response (Figure 3-4).

To detect the potential presence of a countercurrent exchange system for $PGF_{2\alpha}$ between the uterine venous and ovarian arterial vasculature, concentrations of $PGF_{2\alpha}$ were compared between the UBOA and FA. A positive difference in $PGF_{2\alpha}$ concentration between the UBOA and FA would be indicative of an exchange between the OV and UBOA. As

Figure 3-2. Least squares means (□) and regression line depicting the uterine blood flow response to E_2 injection.

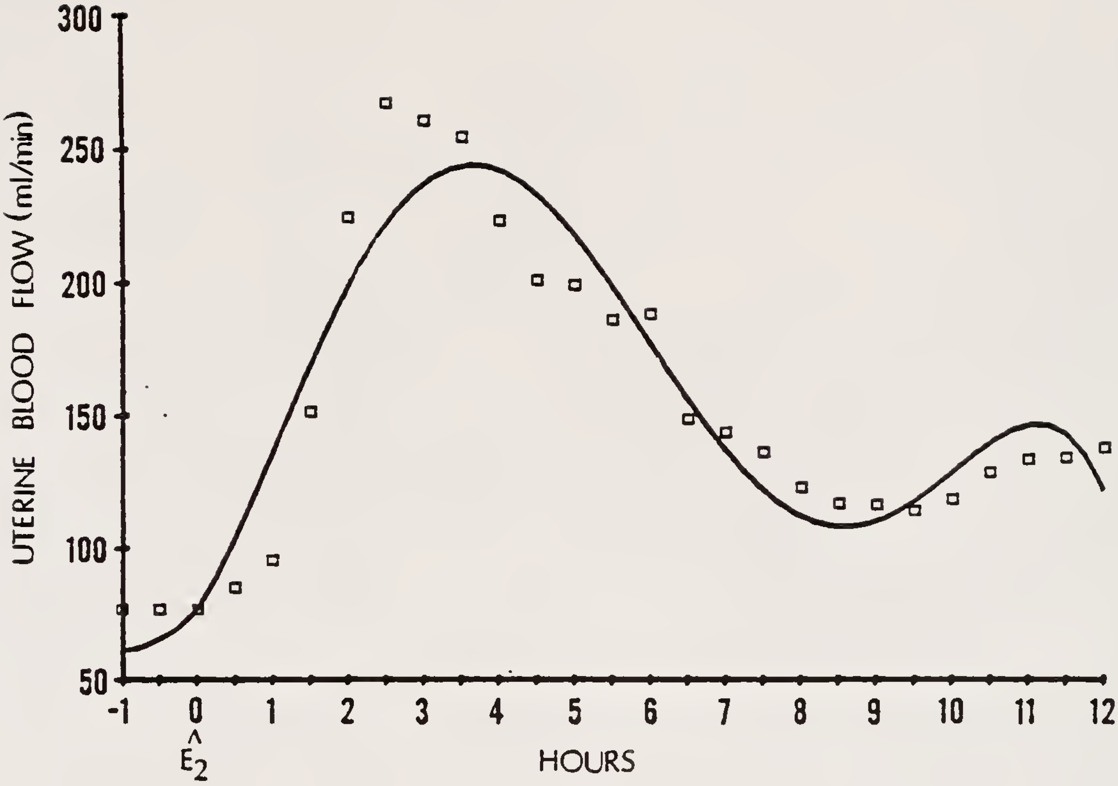

UTERINE BLOOD FLOW (ml/min)
300
250
200
150
100
50
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
$\hat{E}_2$
HOURS

Figure 3-3. Least squares means (symbols) and regression lines for E_2-induced production of $PGF_{2\alpha}$ (Δ, ———), PGFM (□ , -----) and total prostaglandin ($PGF_{2\alpha}$+PGFM; +, —··—) by the uterus.

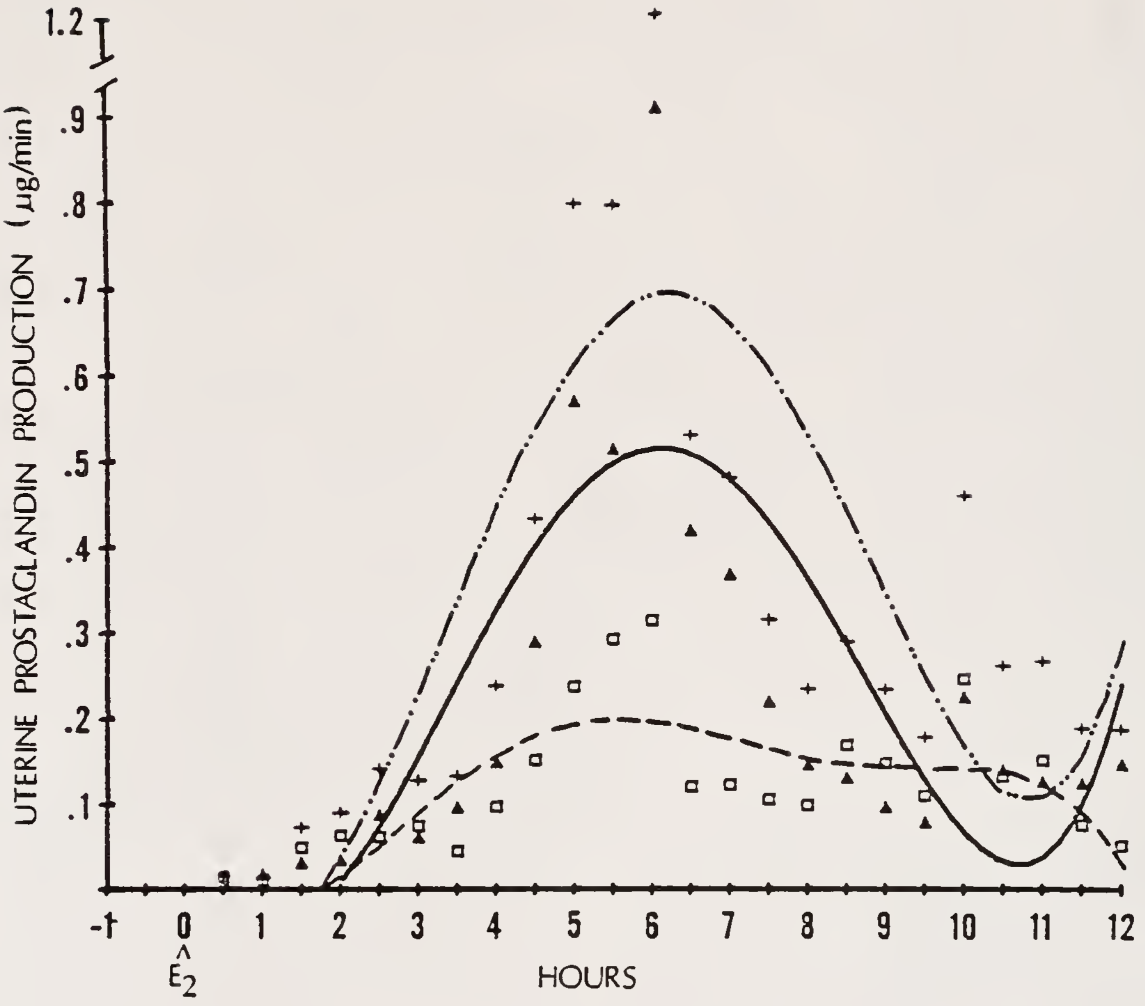

1.2
.9
.8
.7
.6
.5
.4
.3
.2
.1
UTERINE PROSTAGLANDIN PRODUCTION (μg/min)
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
$\hat{E_2}$
HOURS

Figure 3-4. Least squares means (symbols) and regression lines for uterine $PGF_{2\alpha}$ production (+, -----), and peripheral (FA) $PGF_{2\alpha}$ (□) and PGFM (Δ, ———) concentrations.

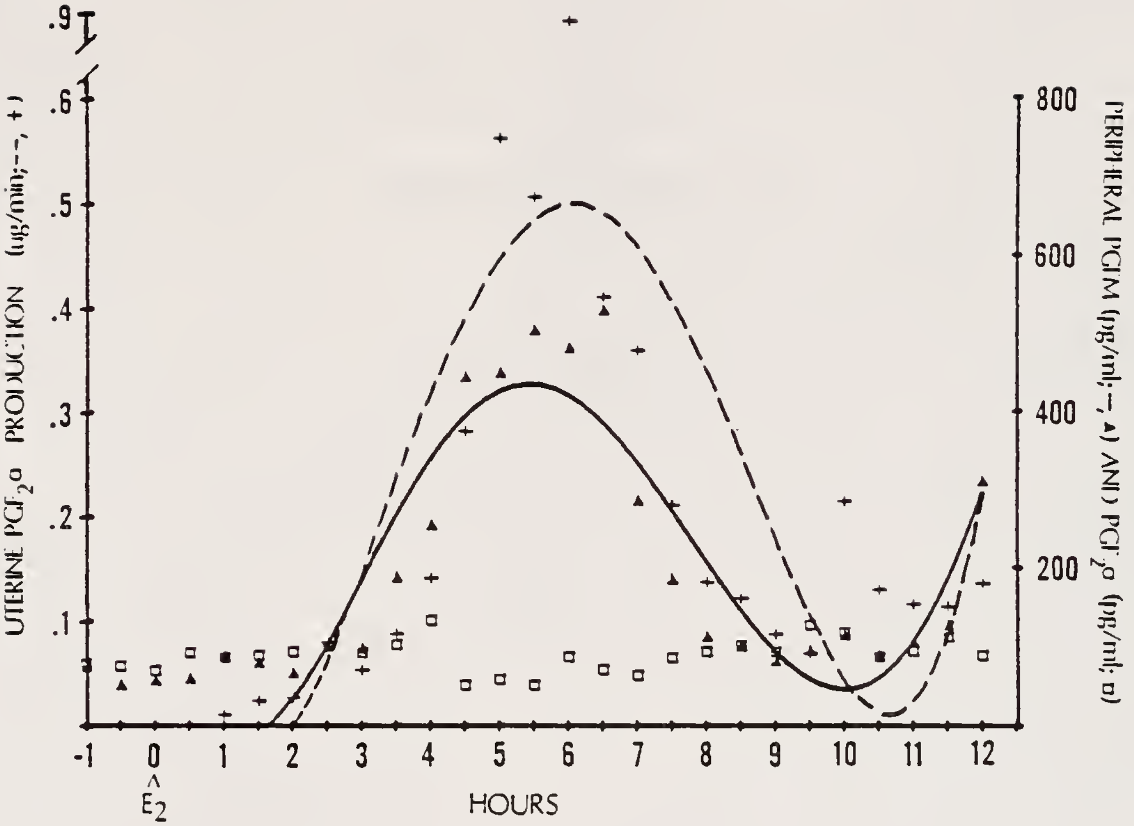

UTERINE PGF$_2\alpha$ PRODUCTION (ug/min;--, +)
PERIPHERAL PGFM (pg/ml;—,▲) AND PGF$_2\alpha$ (pg/ml; □)
.9
.6
.5
.4
.3
.2
.1
800
600
400
200
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
^
E$_2$
HOURS

Table 3-3. Correlations of PGFM in facial artery with uterine $PGF_{2\alpha}$ and PGFM responses

	Gross Correlation*	Within Cow Partial Correlation**
OV-FA $PGF_{2\alpha}$	.52	.64
OV-FA PGFM	.42	.22*
OV-FA $PGF_{2\alpha}$ + PGFM	.53	.55
$PGF_{2\alpha}$ Production	.64	.66
PGFM Production	.42	.30
Total ($PGF_{2\alpha}$ + PGFM) Production	.62	.59

**P<.01
* P<.10

illustrated in table 3-4, concentrations of $PGF_{2\alpha}$ in the UBOA were greater than those in the FA in three of five cows. An utero-ovarian arterial anastomosis (UA to UBOA) was detected below the point of catheter entry in one cow following dissection of the reproductive tract at slaughter. Such a link between the uterine and ovarian arteries may have diluted $PGF_{2\alpha}$ concentrations in the ovarian artery with peripheral blood, low in $PGF_{2\alpha}$, and could explain our inability to demonstrate a countercurrent response in this cow. However, evidence for a countercurrent exchange system was detected in three cows and a significant cow x vessel interaction was detected ($P<.01$). Such a system would require $PGF_{2\alpha}$ to pass down a concentration gradient from plasma of the OV (1382.8 pg/ml; Table 3-1) into the ovarian artery (UBOA-FA, 508 pg/ml; Table 3-1).

Discussion

Results demonstrated that E_2 stimulated an increase in UBF and induced prostaglandin production by the diestrus bovine uterus (day 18). Uterine blood flow increased within 15 to 30 min following E_2 administration reaching maximal values by 2.5 h. Similar UBF responses have been reported for cattle (Roman-Ponce et al., 1978) and sheep (Huckabee et al., 1970; Killam et al., 1973; Barcikowski et al., 1974) following estrogen administration. The mechanisms by which

Table 3-4. Evaluation of differences in $PGF_{2\alpha}$ (pg/ml) between uterine branch of ovarian artery (UBOA) and facial artery (FA) post-E_2 injection

Cow	UBOA	FA
1	2653.7 ± 680.4	50.0 ± 0.0
4	50.0 ± 0.0	50.0 ± 0.0
5[a]	78.9 ± 11.6	124.8 ± 21.6
7	94.0 ± 15.1	54.6 ± 3.7
8	192.3 ± 71.4	50.0 ± 0.0

Cow x Vessel, $P<.001$

[a] An utero-ovarian arterial anastomoses was detected below the point of UBOA catheter entry in this cow (see text).

estrogen induces UBF are not known, however, processes dependent upon protein synthesis may be involved. Induction of UBF by estrogen was completely inhibited during uterine arterial infusion of cyclohexamide (an inhibitor of RNA dependent protein synthesis) in ovariectomized sheep. Inhibition was overcome 25 min following cessation of cyclohexamide treatment (Killam et al., 1973). In contrast, local administration of actinomycin D to the uterus had no effect on the ability of estrogen to increase UBF even though 44 to 74% of DNA dependent RNA synthesis had been inhibited (Resnik et al., 1975). Thus post-translational control of protein synthesis appears to play an essential role in regulating the estrogen induced UBF response.

Ford (1982) and Ford and Reynolds (1983) indicated that vasodilatory effects of estrogen on the uterine vasculature may be mediated through an antagonistic interaction of catechol-estrogens (2 or 4 hydroxylated metabolites of estrogen) on α-adrenergic receptors. Such a hypothesis is supported by 1) the similarity in structure of the catechol-estrogens and the catecholamine, norepinephrine, and 2) initiation of immediate UBF elevations following administration of phentolamine, an α-adrenergic antagonist. A delay of 15 to 30 min for E_2 to induce an increase in UBF, in the present study, may represent the time required for tissue hydroxylation of E_2 and subsequent action of catechol-estrogen on the uterine vasculature.

In contrast to the rapid UBF response, elevations in uterine prostaglandin production were not evident until 2 to 3 h following administration of E_2. A decline in UBF after 3 h occurred simultaneously with the increase in uterine $PGF_{2\alpha}$ production (Figures 3-2 and 3-3). One contributing factor to the reduction in UBF may be an increase in vasculature resistance due to the vasoconstrictive effect of $PGF_{2\alpha}$ on the uterine arterial vasculature (Clark et al., 1973; Ford et al., 1976; Ford, 1982). Time trends, post-E_2 injection, for uterine venous prostaglandin concentrations in the present study (Fig. 3-1) were essentially parallel to uterine prostaglandin production profiles (Fig. 3-3). This demonstrated that prostaglandin concentrations in uterine venous drainage were a function of uterine tissue synthesis and release and not simply alterations due to changing UBF.

Protein synthesis appears to be involved in estrogen stimulated prostaglandin synthesis and release. The 2 to 3 h latency period until uterine prostaglandin production increased may have reflected an estrogen induced protein synthetic process through which uterine $PGF_{2\alpha}$ synthesis depends. French and Casida (1973) demonstrated that the luteolytic effect of estrogen was prevented when uterine protein synthesis was inhibited with actinomycin D. Infusion of E_2 into the arterial supply of autotransplanted ovine uteri induced an increase of $PGF_{2\alpha}$ in the uterine venous drainage (Barcikowski et al., 1974). Prostaglandin

$F_{2\alpha}$ was markedly reduced when a concurrent infusion of E_2 and indomethacin was evaluated. Indomethacin specifically inhibits cyclo-oxygenase, a rate limiting enzyme for synthesis of prostaglandin (Ramwell et al., 1977). Huslig et al. (1979) reported cyclical changes in concentrations of uterine cyclo-oxygenase in ewes which occurred simultaneously with changes in uterine $PGF_{2\alpha}$ secretion. They suggested that exposure of the progesterone primed uterus to estrogen was responsible for synthesis of this rate limiting enzyme.

In addition to stimulation of uterine $PGF_{2\alpha}$ synthesis, E_2 also increased uterine metabolism of $PGF_{2\alpha}$ as demonstrated by the PGFM production profile in the present study (Fig. 3-3). Data provide indirect evidence that estrogen may regulate enzymatic activity responsible for uterine metabolism of $PGF_{2\alpha}$ (i.e., 15-hydroxyprostaglandin dehydrogenase and Δ^{13}-reductase) as well as those for $PGF_{2\alpha}$ synthesis (phospholipase A_2 and cyclo-oxygenase). Rapid metabolism of $PGF_{2\alpha}$ by the lung, uterus and possibly other tissues (Piper et al., 1970; Ramwell et al., 1977; Granstrom and Kindahl, 1982) precludes the parent molecule from being used as a peripheral marker for uterine prostaglandin production. As demonstrated in this study, peripheral concentrations of $PGF_{2\alpha}$ were basal during the pre- and post-E_2 phases (Table 3-1; Fig. 3-4) and were not correlated with any uterine prostaglandin response (Fig. 3-4). In contrast, measurement of the primary metabolite of $PGF_{2\alpha}$, PGFM, in

peripheral plasma was related to uterine $PGF_{2\alpha}$ production ($r=.66$; Fig. 3-4). Measurement of peripheral concentrations of PGFM have been used to monitor uterine $PGF_{2\alpha}$ during the estrous cycle (Peterson et al., 1975; Kindahl et al., 1976), early pregnancy (Kindahl et al., 1976; Betteridge et al., 1984), postpartum (Guilbault et al., 1984a,b) and following estrogen injection (Thatcher et al., 1979; Bartol et al., 1981b; Rico et al., 1981) in cattle. Direct evidence in support of the assumption that peripheral PGFM concentrations reflected endogenous uterine $PGF_{2\alpha}$ production was shown by Bartol et al. (1981b) and is demonstrated clearly in the present study. These results support the use of peripheral PGFM as an index of uterine prostaglandin production in cattle.

The countercurrent exchange of $PGF_{2\alpha}$ from the uterine venous drainage to the ovarian arterial supply (Hixon and Hansel, 1974; Baird, 1978; Ginther, 1981) was examined during a period of E_2-induced $PGF_{2\alpha}$ production. Three of five cows exhibited higher ($P<.01$) concentrations of $PGF_{2\alpha}$ in the UBOA versus FA supporting the existence of such an exchange (Table 3-4). Variations of $PGF_{2\alpha}$ concentrations were greater ($P<.01$) in plasma from the UBOA than FA. This was true regardless of whether cow number one (Table 3-4) was included or deleted from the analyses. Increased variation in $PGF_{2\alpha}$ concentrations in the UBOA suggests that concentrations rose intermittently above those of the FA

during the 12 h sampling period following E_2 injection. These data, in addition to the significant cow x vessel interaction, provide support for the existence of a $PGF_{2\alpha}$ countercurrent exchange system in cattle.

Shemesh and Hansel (1975) were unable to substantiate the existence of a $PGF_{2\alpha}$ countercurrent exchange system in luteal phase, cyclic cattle following the collection of single blood samples at surgery. Data in the present study were based upon numerous blood samples collected from conscious cattle and provides additional support for a countercurrent exchange system. The vasoactive effects of E_2 and $PGF_{2\alpha}$ on the uterine and ovarian vasculature and their potential influence on efficiency of the countercurrent exchange warrant additional investigation.

In summary, exogenous E_2 induced an increase in UBF and uterine prostaglandin production and metabolism. Measurement of peripheral PGFM concentrations was a good index of uterine prostaglandin production. Three of five cows demonstrated a countercurrent exchange of $PGF_{2\alpha}$ during a period of E_2-induced $PGF_{2\alpha}$ production.

The E_2 injection scheme may provide a good experimental model with which to investigate uterine prostaglandin synthetic and metabolic capacity during various physiological states in cattle and a means to study conceptus-uterine interactions during the period of maternal recognition of pregnancy.

CHAPTER 4
PROTEINS SECRETED BY DAY 16 TO 18 BOVINE CONCEPTUS EXTEND CORPUS LUTEUM FUNCTION IN CATTLE

Introduction

An essential manifestation of pregnancy in large domestic species is extended corpus luteum (CL) function. During the first 15-16 days of pregnancy in cattle, progesterone (P_4) production by the CL establishes a complex uterine environment essential for conceptus (embryo plus extraembryonic membranes) growth and development. Embryos may be transferred, and pregnancies established as late as day 16 or 17 post-estrus (Betteridge et al., 1980). Consequently, presence of a conceptus within the uterine lumen prior to day 16 is not a requirement for initiation of an embryotrophic uterine environment. Beyond this point, however, a viable conceptus within the uterine lumen must play an active role in the perpetuation of its embryotrophic environment by maintenance of the CL (Betteridge et al., 1980; Northey and French, 1980; Dalla Porta and Humblot, 1983).

The bovine conceptus produces an array of potential "signals" during early pregnancy including steroids (Shemesh et al., 1979; Chenault, 1980; Gadsby et al., 1980; Eley et

al., 1983), prostaglandins (Shemesh et al., 1979; Lewis et al., 1982) and proteins (Bartol et al., 1984). However, conceptus factors responsible for luteal maintenance during early pregnancy and their mechanisms of action have not been demonstrated clearly in cattle. Numerous studies have evaluated the effect of prostaglandin (PG)-E_2 on luteal maintenance in cattle with mixed results. Prostaglandin-E_2 administered into the uterine lumen alone (Chenault, 1983; Gimenez and Henricks, 1983) or in combination with estradiol-17β (Reynolds et al., 1983) extends luteal function only slightly beyond the cessation of intrauterine PGE_2 treatments. In addition, systemic P_4 concentrations declined within 12 h following PGE_2 treatment (Gimenez and Henricks, 1983) or during the treatment period (Reynolds et al., 1983) suggesting that PGE_2 (plus estradiol-17β) will not prevent production and transfer of uterine luteolytic substances, but affects luteal function directly (Marsh, 1970; Henderson et al., 1977; Reynolds et al., 1981). Others have reported no affect of intrauterine PGE_2 administration on CL maintenance in cattle (Dalla Porta and Humblot, 1983; Chenault et al., 1984).

Extension of CL maintenance and cycle length were demonstrated following intrauterine administration of conceptus homogenates to cyclic cattle (Northey and French, 1980). Likewise, ovine conceptus extracts and homogenates (Rowson and Moor, 1967; Ellinwood et al., 1979; Martal et

al., 1979) or conceptus secretory proteins (Godkin et al., 1984) have been shown to extend CL function and cycle length when administered into the uterine lumen of cyclic ewes. However, luteotropic and/or antiluteolytic actions of major conceptus-produced steroids and proteins have not been evaluated in cattle. Objectives of the present experiment were to examine the effects of a major bovine conceptus-produced steroid (Eley et al., 1983; Chapter 2), 5β-pregnan-3α-ol-20-one, and bovine conceptus secretory proteins on luteal function, cycle length and spontaneous uterine $PGF_{2\alpha}$ production in cyclic cattle.

Materials and Methods

Conceptus Collection and Culture

Dairy and beef cows (n=49) served as conceptus donors after being mated during estrus (day 0). Between days 16 to 18 post-mating (17.2 ± 0.6 days), the uterine horn ipsilateral to the corpus luteum (CL) bearing ovary was nonsurgically flushed. A French Foley catheter (size no. 16, 18 or 20; American Hospital Supply, Jacksonville, FL) was inserted through the cervical os and positioned at the base of the uterine horn just anterior to the uterine body. Sterile Dulbeccos phosphate buffered saline (pH 7.4; PBS; Dulbecco & Vogt, 1954) was warmed to 37 C and injected into the uterine lumen (I.V.) at a volume of 60 ml per

flush. Medium containing conceptuses (n=33) was collected into sterile glass containers, maintained at 37 C, and transported to the laboratory within 30 min of recovery. Following the last uterine flush, 20 ml of an antibiotic solution (aqueous Procaine Penicillin G; 300,000 units/ml; Pfizer Incorporated; New York, NY) were infused into the uterine lumen, and Foley catheter removed. Donor cows were bred and flushed up to three times during successive estrous cycles.

An additional four cows were superovulated, bred and slaughtered at 17 days post-mating. The uterus was removed following exsanguination, sealed in a plastic bag, and placed on ice while being transported to the laboratory (within 60 min of slaughter). Uterine horns were trimmed of excess tissue, ovaries and oviducts removed, and a large, curved, Rochester-Ochsner forcep applied to the anterior cervix. The anterior tip of the uterine horn ipsilateral to the CL containing ovary was cut to provide an enlarged opening. A plastic, 50 ml syringe fitted with an 18 gauge needle was used to administer two 30 ml flushes (sterile PBS, warmed to 37 C) into the uterine lumen through the tip of the uncut uterine horn. Medium containing conceptuses was collected into sterile plastic culture dishes. Approximately 15 to 20 conceptuses (n=15 incubations) were obtained from superovulated animals. All conceptuses, collected either nonsurgically or at post-mortem flush, were

washed in and transferred to sterile culture dishes containing 15 ml of warm Minimum Essential Medium (MEM; GIBCO, Grand Island, NY) supplemented with non-essential amino acids (GIBCO, Grand Island, NY), antibiotic/antimycotic (GIBCO, Grand Island, NY), 200 units of insulin/L (Sigma Chemical Co., St. Louis, MO) and 1 g glucose/L (Fischer Scientific; Orlando, FL). Conceptuses were cultured for 24 h on a rocker platform (Bellco Glass Company, Vineland, NJ) and maintained at 37 C in a gaseous environment of nitrogen:oxygen:carbon dioxide (50:45:5). Following a 24 h incubation, tissues and medium were separated by centrifugation (10,000 x g; 20 min) at 4 C. Medium (supernatant) from each culture was collected and frozen individually. Conceptus wet weights were recorded.

Preparation of Material for Intrauterine Injections

Medium from individual incubations (15 ml) were dialyzed (Spectrapore 6, 1000 Mr cutoff, Spectrum Medical Industries, Los Angeles, CA) extensively (4 L changed thrice daily for 5 days) against 10 mM Tris-HCl buffer, pH 7.2 (TRIS). Following dialysis, an aliquot of medium from each culture (n=47) was used for determination of protein concentration (Lowry et al., 1951). All culture medium was then pooled and concentrated by ultrafiltration (1000 Mr cutoff; Amicon Corporation, Danvers, MA) to a volume of approximately 75 ml. The concentrated filtrate was processed through a sterilization filter unit (0.45 µm pore

size; Sybron/Nalgene, Rochester, NY) and dispensed into 2 ml aliquots designated as pooled conceptus secretory proteins (CSP) at a protein concentration of 740 μg/2 ml.

A serum sample from each experimental animal (n=9) was collected on day 10 of the estrous cycle. Serum samples were dialyzed individually, diluted in TRIS and sterilized as described for CSP. Equal masses (740 μg) of homologous serum proteins were added to each 2 ml injection of CSP (n=3 cows; 12 injections/cow; Group 1).

Two milligrams of 5β-pregnan-3α-ol-20-one (5β-P) were dissolved in 2 ml of ethanol and mixed with 80 ml of TRIS. Ethanol was evaporated from the solution using N_2 gas and gentle heating. The steroid solution was sterilized as described previously and dispensed into 2 ml aliquots (50 μg each). Homologous serum proteins (740 μg) were added to each 2 ml injection of 5β-P (n=3 cows; 12 injections/cow; Group 2). Group 3 cows (n=3) received homologous serum proteins alone (1480 μg/2 ml TRIS; 12 injections/cow). All treatment mixtures were frozen (-20 C) until time of intrauterine injection.

Animal Preparations

Nine cyclic Holstein cows were assigned randomly to the three previously described treatment groups. Animals were prepared for surgery (Chapter 3) on day 10 of the estrous cycle. Utilizing a midventral laparatomy, uterus and ovaries were exposed and location of the CL recorded. A

sterile polyvinyl catheter (V-6; Bolab Incorporated, Lake Havasu City, AZ) was inserted via an incision in the isthmus of the oviduct and secured 30 to 50 mm into the anterior portion of the uterine lumen, ipsilateral to the CL. The catheter was exteriorized via a small flank incision. An additional catheter (Sialastic tubing, I.D. 1.57 mm x O.D. 3.18 mm; Dow Corning Corporation, Midland, MI) was advanced approximately 1.07 m into the saphenous vein to a position in the dorsal vena cava slightly anterior to the point of uteroovarian venous drainage. Jugular catheterizations (V-9; Bolab Incorporated, Lake Havasu City, AZ) were performed, if necessary, at the time of vena cava catheter failure during the experiment. Antibiotics (Polyflex, 167 mg/ml; Bristol Laboratories, Syracuse, NY) were administered on the day of surgery (10 ml I.M., 10 ml I.P. and 1 ml I.U.) and one day post-surgery (10 ml I.M. and 1 ml I.U.).

Function of the CL was monitored by measuring plasma P_4 concentrations from blood samples collected twice daily (0800 and 2000 h) beginning on day 12 and continuing until detection of oestrus. Three acute bleedings were conducted to monitor spontaneous uterine prostaglandin (PG) production. Plasma concentrations of $PGF_{2\alpha}$ were measured in blood samples drawn every 15 min from 0800 to 1400 h on days 18 through 20. Intrauterine injections of experimental materials (2 ml; see above) were initiated at 2000 h on day 15 and continued every 12 h until 0800 h of day 21. All

animals were fitted with oestrous-mount detection devices (KaMar Incorporated, Steamboat Springs, CO), maintained on pasture in the presence of an intact bull, and observed two to four times daily for oestrous behavior. Corpus luteum regression was verified by rectal palpation post-oestrus. When the experiment had concluded, all animals were slaughtered and reproductive tracts dissected to assess uteroovarian condition and catheter placement.

Progesterone and Prostaglandin Radioimmunoassay

Progesterone (P_4) was measured in heparinized plasma samples utilizing a specific antiserum generated in sheep against P_4 conjugated to bovine serum albumin (BSA) at the C^{11}-position. Tritiated P_4 ([1,2,6,7-^{3}H(N)], specific activity 90-115 Ci/mM) was purchased from New England Nuclear (Boston, MA). Cross-reactivity of the P_4 antiserum was < 1% with 17α-hydroxy-P_4, 20α-hydroxy-P_4, 20β-hydroxy-P_4, cortisol, testosterone, androstenedione and estradiol-17β. Standard curves were prepared by adding known amounts of radioinert P_4 to phosphate buffered saline (pH 7.4) containing 1 g/L of gelatin (PBSg). Final range of P_4 concentrations were: 0, 15.6, 31.2, 62.4, 125, 250, 500 and 1000 pg/100 µl of PBSg. Utilizing an antiserum dilution of 1:35,000, sensitivity of the assay was 15.6 pg. A plasma sample containing approximately 4.5 ng/ml of immunoreactive P_4 was aliquoted in triplicate into sample volumes of 50,

100, 200 and 300 µl. Progesterone from plasma samples were extracted by vortexing for 2 min with 2 ml of freshly distilled benzene and hexane (1:2). Solvent containing the extracted P_4 was dried under N_2 and brought to a 500 µl assay volume with the addition of PBSg. A quantitative linear recovery was achieved [$\hat{Y}$ = 20.965 + 4.111X; $\hat{Y}$ = concentration of P_4 (pg/100 µl) and X = plasma volumes extracted (µl)]. No significant differences were found between concentrations of P_4 (pg/100 µl) measured using either 50, 100, 200 or 300 µl aliquot volumes ($P>0.25$). Exogenous P_4 was added to a plasma sample ($\bar{X}$ = 1.8 ng/ml) at doses of 0, 0.5, 1.0, 5.0 and 10 ng/ml. All doses were replicated four times. A linear regression equation of added vs measured P_4 [$\hat{Y}$ = 1626.1 + 1.0855X; $\hat{Y}$ = amount of P_4 measured (pg/ml) and X = amount of P_4 added (pg/ml); R^2 = 0.975] described differences among concentrations. The intra-assay coefficient of variation (CV) for the validation was 13.4%. Intra- and interassay CV for eight assays were: 10.4 and 7.5%, respectively, when duplicate estimates were run in the assays for a 100 pg/500 µl (94.2 ± 4.3 pg/500 µl) reference plasma sample, and 6.1 and 4.3%, respectively, when duplicate estimates were run for a 250 pg/500 µl (269.5 ± 7.0 pg/500 µl) references plasma sample.

Prostaglandin-$F_{2\alpha}$ was assayed in unextracted plasma samples (50, 100 and 200 µl). Concentrations of $PGF_{2\alpha}$ were determined in a dextran coated charcoal radioimmunoassay

system previously validated for use in our laboratory (Knickerbocker et al., 1982; Chapter 3). Antiserum used in the assay was generated in goats against $PGF_{2\alpha}$ conjugated to BSA at the C^1-position. This antiserum crossreacts 53.6% with $PGF_{1\alpha}$ and < 1% with 15-keto-$PGF_{2\alpha}$, 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM), PGE_1, PGE_2 and arachidonic acid. Sensitivity of the assay was 10 pg. The intra- and inter-assay CV for 13 assays were: 20.0 and 12.7%, respectively, when duplicate estimates were run for a 50 pg/200 μl (60.9 ± 2.5 pg/200 μl) plasma reference, and 10.6 and 5.6%, respectively, when duplicate estimates were run for a 500 pg/200 μl (489.9 ± 10.1 pg/200 μl) plasma reference.

Statistical Analyses

Data for P_4 and $PGF_{2\alpha}$ concentrations in plasma, and accumulated $PGF_{2\alpha}$ were analyzed using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Incorporated, 1982) for a split-plot analysis of variance with repeated measurements over time. Analysis of variance considered variability due to treatment (CSP, 5β-P and Control), cow nested within treatment, time (for P_4: days 12-38.5; for $PGF_{2\alpha}$: samples 1-75 which consisted of 25 samples on each of days 18-20), treatment by time, and cow nested within treatment by time. Estimates for $PGF_{2\alpha}$ produced over the 6 h sampling periods on days 18 through 20 were generated by sequential summation of $PGF_{2\alpha}$ concentrations measured in plasma on days 18 through 20.

Using this method, a series of $PGF_{2\alpha}$ values were generated over days for each sample within cow. Each $PGF_{2\alpha}$ value corresponded to the $PGF_{2\alpha}$ concentration in the respective sample plus a summation of $PGF_{2\alpha}$ concentrations in all prior samples (accumulated $PGF_{2\alpha}$). To provide estimates of time trends, P_4 concentrations and accumulated $PGF_{2\alpha}$ values were analyzed by least squares regression analyses, and differences in treatment means evaluated by orthogonal comparisons (CSP versus 5β-P and Control; 5β-P versus Control). Differences in polynomial regressions were tested by examining for homogeneity of regression between treatment response curves. These data were analyzed with time as a continuous, independent variable. Data pertaining to cycle length, days with P_4 concentrations > 1 ng/ml, and mean accumulated $PGF_{2\alpha}$ (Table 4-1), as well as age effects for conceptus wet weight, and µg protein produced/mg conceptus wet weight (Table 4-2) were analyzed by a one-way analysis of variance. Treatment differences were evaluated by orthogonal comparisons (refer to appropriate tables for information on the specific orthogonal comparisons).

Results

Evaluation of Reproductive Tracts

Cows were palpated per rectum following detected oestrus. In all cases, a single regressing CL was detected

Table 4-1. Effects of intrauterine treatments on cycle length, luteal function and $PGF_{2\alpha}$ production[ab]

Treatment	Cycle length	Days with P_4 >1 ng/ml	Accumulated $PGF_{2\alpha}$ pg/cow/day (n)
CSP	33.42±2.49**	30.33±1.85**	39.17± 35.75(6)**
5β-P	24.67±0.83	22.67±1.01	1322.44±544.42(7)**
Control	23.50±0.50	22.33±0.60	496.41±210.75(8)

a Mean ± S.E.M.
b Orthogonal comparisons: CSP vs. 5β-P and Control; 5β-P vs. Control.
** P<0.01.

Table 4-2. Bovine conceptus wet weight and protein production[ab]

Age (days)	N	Wet Weight (mg)[c]	μg Protein/mg Wet Weight[d]
16.5	9	31.74±7.73	4.93±2.39*
17.0	13	35.08±3.63	15.76±5.17
17.5	4	27.58±7.99	12.04±4.07
18.0-18.5	6	55.78±8.55*	15.88±5.25

a Data from conceptuses collected following superovulation are not included in analysis (n=15 cultures; 22.30±1.97 μg protein/mg wet weight).
b Mean ± S.E.M.
c Orthogonal comparison: 18.0-18.5 vs. 16.5, 17.0, 17.5.
d Orthogonal comparison: 16.5 vs. 17.0, 17.5, 18.0-18.5.
* P<0.05.

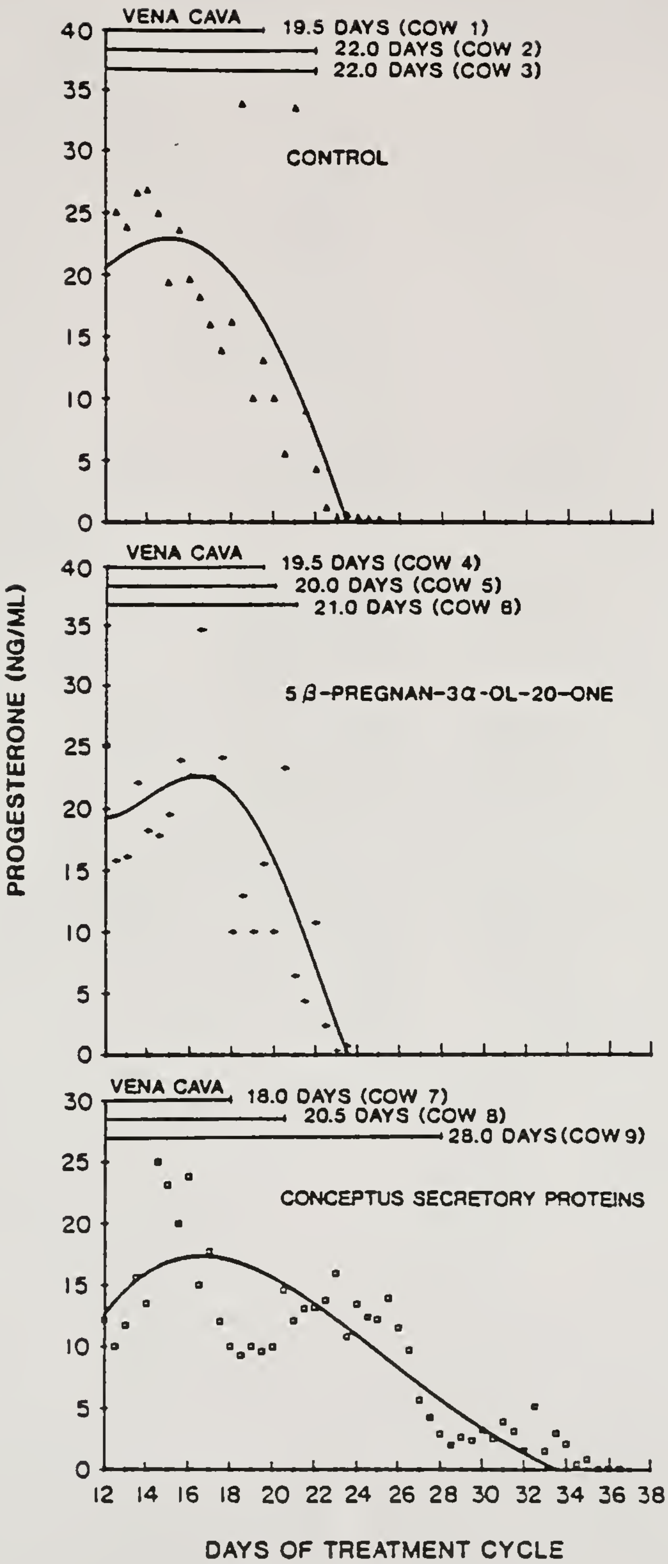
on the same ovary bearing a functional CL at surgery. Upon dissection of reproductive tracts post-slaughter, all catheters were intact and patency was verified. General appearance of endometrium and uteroovarian tissues were normal for all experimental animals.

Effects of Intrauterine Injections on Interestrous Interval and Corpus Luteum Function

Estrous cycle lengths immediately preceding the experimental estrous cycle were not different ($P>0.25$) among cows assigned to CSP, 5β-P and Control groups (20.57 ± 0.89 days). Intrauterine administration of CSP to cyclic cows resulted in extended estrous cycle lengths ($P<0.01$) of 30, 31.75 and 38.5 days as compared to cows which received 5β-P (23, 25.5 and 25.5 days) and Control (22.5, 24 and 24 days) injections. No differences were detected ($P>0.25$) in interestrous intervals between cows of the 5β-P and Control groups (Table 4-1).

Analysis of plasma P_4 concentrations verified observations associated with rectal palpations and interoestrous intervals (Fig. 4-1, Table 4-1). Function of the CL (P_4 concentrations > 1 ng/ml) was maintained for 28, 29 and 34 days following CSP treatments, whereas CL lifespan of cows in the 5β-P (21, 22.5 and 24.5 days) and Control (21.5, 22 and 23.5 days) groups were shorter ($P<0.01$). A major conceptus-produced steroid, 5β-pregnan-3α-ol-20-one, did not influence CL lifespan as compared to Control cows treated with homologous serum proteins alone. Vena cava catheters

Figure 4-1. Least squares means and regressions of plasma progesterone concentrations for groups of cows ($n=3$ per group) receiving intrauterine injections (days 15.5 to 21) of homologous serum proteins (control), 5β-pregnan-3α-ol-20-one, or conceptus secretory proteins. Horizontal lines above each graph represents duration of vena cava catheter patency in each cow.

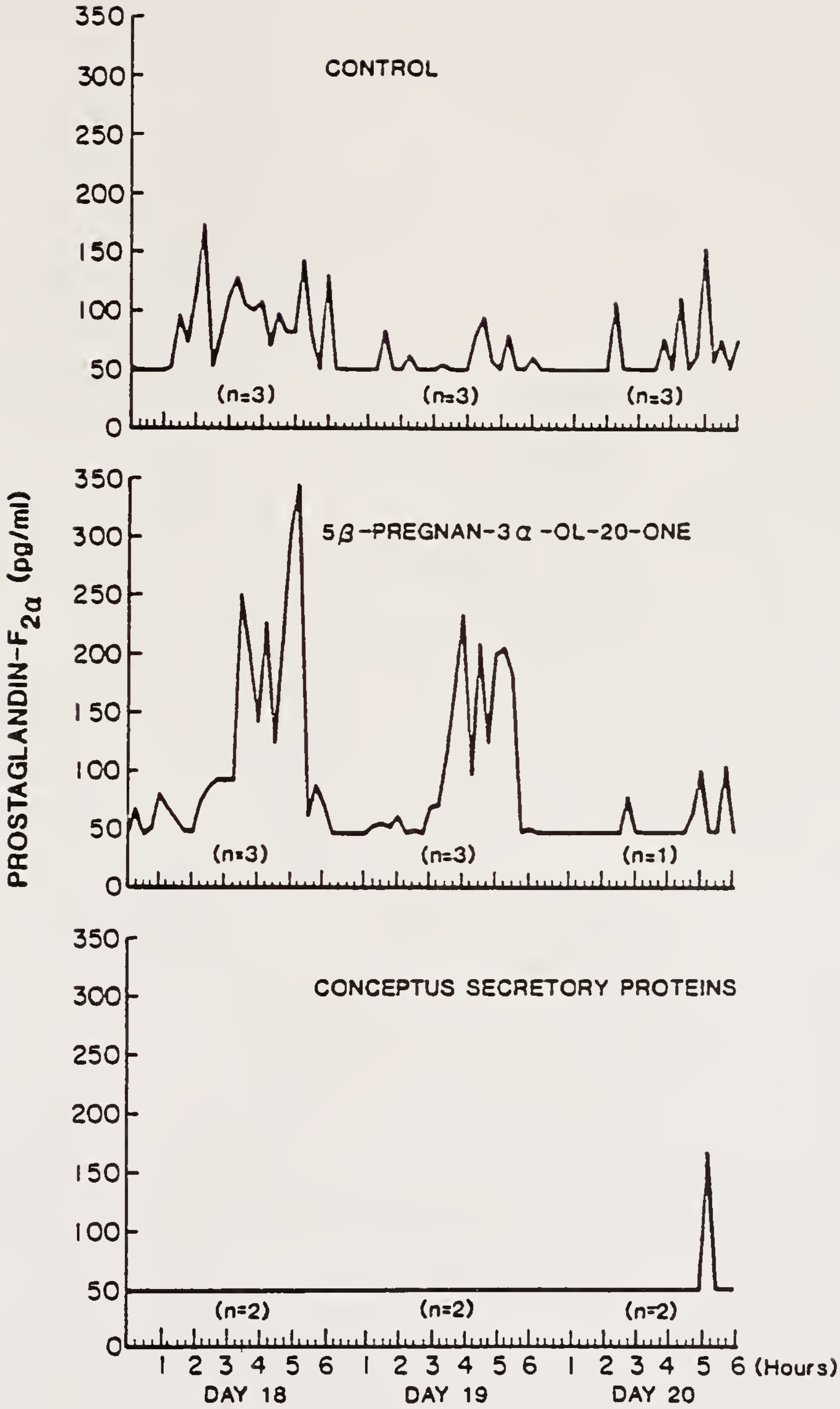

VENA CAVA
19.5 DAYS (COW 1)
22.0 DAYS (COW 2)
22.0 DAYS (COW 3)
CONTROL
VENA CAVA
19.5 DAYS (COW 4)
20.0 DAYS (COW 5)
21.0 DAYS (COW 6)
5 β-PREGNAN-3α-OL-20-ONE
PROGESTERONE (NG/ML)
VENA CAVA
18.0 DAYS (COW 7)
20.5 DAYS (COW 8)
28.0 DAYS (COW 9)
CONCEPTUS SECRETORY PROTEINS
DAYS OF TREATMENT CYCLE

were maintained to approximately day 21 of the experimental cycle (21.17 ± 1.05; Fig. 4-1) at which time jugular catheters were installed (eight of nine cows). Following loss of vena cava catheter patency, seven of eight experimental cows exhibited jugular concentrations of P_4 ($\bar{X}$ ± S.E.) in excess of 1 ng/ml (6.1 ± 1.8 ng/ml), which indicated presence of a functional CL. Concentrations of P_4 were two to ten times higher in vena cava plasma immediately prior to failure of the vena cava catheter (17.5 ± 6.3 ng/ml) versus concentrations in jugular plasma.

Effects of Intrauterine Injections on Plasma Prostaglandin Concentrations

Analysis of prostaglandin responses suggested that proteins secreted by bovine conceptuses reduced uterine production of $PGF_{2\alpha}$ (Fig. 4-2). Circulating $PGF_{2\alpha}$ exhibits a relatively short half-life (7-8 min; Kindahl et al., 1976) due to its rapid metabolism by the lung and peripheral tissues (Granstrom and Kindahl, 1982). Therefore, plasma samples from the vena cava were utilized for $PGF_{2\alpha}$ determinations. Higher plasma concentrations of P_4 in vena cava versus jugular vein samples were used as verification of catheter placement prior to $PGF_{2\alpha}$ data analysis. The number of cows contributing to $PGF_{2\alpha}$ responses in vena cava plasma for each group on days 18, 19 and 20 were CSP, 2,2,2; 5β-P, 3,3,1; and Control, 3,3,3, respectively. A significant treatment by time interaction ($P<0.05$) was detected for plasma $PGF_{2\alpha}$ concentrations in the vena cava

Figure 4-2. Least squares means of vena cava $PGF_{2\alpha}$ concentrations. Blood samples were collected every 15 minutes for 6 hours on days 18, 19 and 20. Cyclic cattle received intrauterine injections of homologous serum proteins (control), 5β-pregnan-3α-ol-20-one or conceptus secretory proteins from days 15.5 to 21.



PROSTAGLANDIN-F2α (pg/ml)
CONTROL
5β-PREGNAN-3α-OL-20-ONE
CONCEPTUS SECRETORY PROTEINS
350
300
250
200
150
100
50
0
(n=3)
(n=3)
(n=3)
(n=3)
(n=3)
(n=1)
(n=2)
(n=2)
(n=2)
1 2 3 4 5 6 1 2 3 4 5 6 1 2 3 4 5 6 (Hours)
DAY 18
DAY 19
DAY 20

and supported a role for CSP in the reduction of $PGF_{2\alpha}$ production. Pulsatile episodes of $PGF_{2\alpha}$ concentrations in the vena cava were apparent in plasma samples collected from all cows of the 5β-P treatment group during days 18 through 20. Similarly, spontaneous elevations of $PGF_{2\alpha}$ in vena cava plasma were detected in two of three, three of three, and one of two Control cows on days 18, 19 and 20, respectively. In contrast, no measurable $PGF_{2\alpha}$ was detected in vena cava plasma from two of two cows in the CSP treatment group on days 18 and 19. One plasma sample did contain measurable amounts of $PGF_{2\alpha}$ in one of two cows on day 20. Profiles of $PGF_{2\alpha}$ concentrations (least squares means) for each group are depicted in Fig. 4-2. Concentrations of $PGF_{2\alpha}$ also were accumulated over the 3 days sample and analyzed statistically. Orthogonal comparisons of treatment means indicated that intrauterine administration of CSP resulted in a mean accumulation per cow of less $PGF_{2\alpha}$ than cows in 5β-P and Control treatments (Table 4-1; $P<0.01$). Additionally, accumulation of $PGF_{2\alpha}$ was greater in cows administered 5β-P compared to cows which received homologous serum proteins alone ($p<0.01$).

Bovine Conceptus Observations

Thirty-two conceptuses were classified according to their age (days 16.5, 17, 17.5 and 18-18.5) at the time of nonsurgical flushing (Table 4-2). Although conceptus wet weights did not increase significantly until day 18.0 to

18.5 post-insemination, tissue secretory activity (μg protein produced/mg conceptus wet weight) increased approximately three-fold in conceptuses 17 days and older ($P<0.05$).

Discussion

Maternal recognition of pregnancy occurs by day 16 to 17 in cattle (Betteridge et al., 1980; Northey and French, 1980; Dalla Porta and Humblot, 1983). This represents a critical period during early gestation when production of conceptus signals becomes essential to luteal maintenance and continued endometrial secretory activity (histotroph). A phase of rapid conceptus elongation and differentiation is intimately associated with this period of signal transmission by the bovine conceptus (Chang, 1952; Greenstein et al., 1958; Greenstein and Foley, 1958a,b). Developmental stage of the conceptus may therefore determine the timing of conceptus signal production and secretion during early pregnancy (Bartol et al., 1984).

In the present study, total protein production per mg of conceptus tissue increased after the initiation of conceptus elongation on day 16. This increase in protein secretory activity was observed prior to any detectable increase in conceptus mass (Table 4-2). Conceptus mass may not be an appropriate index of conceptus expansion since

Geisert et al. (1982) concluded that the initial rapid elongation of the porcine conceptus was due to cellular remodelling and not hyperplasia, whereas subsequent conceptus elongation was associated with increasing DNA and RNA content. In bovine conceptus incubations, [^{3}H]-leucine incorporation into nondialyzable secretory products (presumably proteins) supported an increase in production of labelled proteins from days 16 to 19 and 22 (Bartol et al., 1984). Thus, an increase in conceptus tissue secretory activity occurs concomitantly with bovine conceptus elongation.

Maintenance of CL function in cattle is thought to involve both luteotrophic and antiluteolytic processes which are initiated by the conceptus during early pregnancy. Del Campo et al. (1980) provided convincing evidence for the presence of a blood-borne luteotrophic and/or luteal protective factor(s) originating from the pregnant uterine horn during early gestation in cattle. Furthermore, their results support a local, countercurrent transfer of this substance(s) from the venous drainage of the pregnant uterine horn into the adjacent ovarian arterial supply, ipsilateral to the CL.

Uterine-dependent luteolysis in nonpregnant cattle also involves an ipsilateral, venoarterial pathway (Mapletoft et al., 1976). Prostaglandin-$F_{2\alpha}$ is the presumed uterine luteolysin in cattle and numerous other species (Horton and

Poyser, 1976). However, participation of other active $PGF_{2\alpha}$ metabolites (Milvae and Hansel, 1983) and products of the lipoxygenase pathway (Milvae and Hansel, 1984) in bovine luteolysis is possible. In cattle, elevated concentrations (Shemesh and Hansel, 1975) and high amplitude pulses (Nancarrow et al., 1973) of $PGF_{2\alpha}$ in uterine venous blood, and PGFM in peripheral blood (Peterson et al., 1975; Kindahl et al., 1976) are temporally associated with the decline in plasma P_4 concentrations during luteal regression. During early pregnancy, the bovine conceptus may exert an antiluteolytic effect within the uterus since peripheral PGFM pulses are reduced or abolished in pregnant heifers (Kindahl et al., 1976; Betteridge et al., 1984). Similarly, acute elevations in $PGF_{2\alpha}$ urinary metabolites associated with luteal regression were absent during early pregnancy in heifers (Harvey et al., 1984; Plante et al., 1984). However, basal levels of plasma PGFM (Williams et al., 1983) and $PGF_{2\alpha}$ metabolites in urine (Harvey et al., 1984; Plante et al., 1984) are elevated by days 17 to 20 of gestation. Evidence that blood flow is increased to the gravid uterine horn (Ford et al., 1979) and that bovine conceptuses are capable of significant prostaglandin production during early gestation (Shemesh et al., 1979; Lewis et al., 1982), suggest that elevated basal prostaglandin metabolite concentrations observed in pregnant cattle may result from

conceptus production of $PGF_{2\alpha}$ which is transported out of the gravid uterus during early pregnancy. However, this increase in basal prostaglandin concentration is not of sufficient magnitude to initiate luteolysis. Additional support for conceptus-derived antiluteolytic effects in cattle was demonstrated recently. Intravenous injection of estradiol-17β stimulates uterine production of $PGF_{2\alpha}$ which may be accurately indexed by measurements of PGFM in peripheral plasma (Knickerbocker et al., 1982; Chapter 3). This experimental model was utilized to evaluate uterine capacity to produce $PGF_{2\alpha}$ on day 18 of the oestrous cycle and days 18 and 20 of pregnancy (Thatcher et al., 1984b). Estradiol-induced uterine prostaglandin production was reduced significantly in pregnant cows on days 18 and 20. Furthermore, bovine endometrium at day 17 of pregnancy produced substantially less $PGF_{2\alpha}$, in vitro, than endometrium collected on day 17 of the oestrous cycle (Thatcher et al., 1984b). However, no differences in PGE_2 production, in vitro, were apparent between pregnant and cyclic endometrium.

Based on the P_4 profiles (Fig. 4-1, Table 4-1) and $PGF_{2\alpha}$ data (Fig. 4-2, Table 4-1), conceptus secretory proteins appeared not to stimulate luteal synthesis of P_4 (luteotrophic), but allowed for extended luteal function via local suppression of spontaneous uterine $PGF_{2\alpha}$ production (antiluteolytic). Results of a more recent study

demonstrated a significant reduction in estradiol-induced uterine $PGF_{2\alpha}$ production following intrauterine administration of bovine CSP to cyclic cows (Chapter 5). Collectively, these data indicate that proteins secreted by the bovine conceptus are involved in mediation of anti-luteolytic effects during early pregnancy. Reduction in uterine luteolytic activity by conceptus secretory proteins may involve regulation at several levels of the arachidonic acid metabolic cascade (Milvae and Hansel, 1984; Thatcher et al., 1984b), control of endometrial oxytocin and steroid receptor populations (McCracken et al., 1981; McCracken, 1984) or stimulation of endometrial prostaglandin inhibitors (Wlodawer et al., 1976; Shemesh et al., 1984).

In this study, protein signals secreted in culture by elongating bovine conceptuses (day 16.5 through 18.5) extended luteal function and interestrous interval to approximately 30 and 33 days, respectively, when administered into the uterine lumen of cyclic cattle on days 15 through 21 post-estrus. Similar data were reported recently (Godkin et al., 1984) following intrauterine injection of conceptus secretory proteins to cyclic ewes. Intrauterine administration of a prominent, low molecular weight (Mr), acidic polypeptide, termed ovine trophoblast protein-1 (oTP-1), also extended luteal function in cyclic ewes. Thus, oTP-1 may be the primary protein responsible for luteal maintenance in sheep. Roles of specific proteins

secreted by the bovine conceptus have not been examined. However, several recent reports suggest that there may be similarities in the nature and function of conceptus protein signals in cattle and sheep during early pregnancy.

Major components of the protein synthesized and released in culture by day 16 to 24 bovine conceptuses are low Mr, acidic polypeptides (Bartol et al., 1984) similar in nature to those produced by the elongating ovine conceptus (day 13-21); Martal et al., 1984b; Godkin et al., 1982b). When bovine (day 14) and ovine (day 11-13) trophoblastic vesicles (TV), composed of extraembryonic trophectoderm and endoderm, were transferred and allowed to develop in cyclic cattle and sheep, respectively, a majority of the recipients exhibited prolonged CL maintenance (Heyman et al., 1984; Martal et al., 1984b). These data support previous reports (Godkin et al., 1982b, 1984) that the extraembryonic trophectodermal layer of the conceptus secretes proteinaceous signals responsible for CL maintenance in early pregnancy. In a related study (Martal et al., 1984a), cross-species transfers of bovine and ovine TVs led to extended CL function in approximately 20% of ovine and bovine recipients. The authors suggested that nonspecific conceptus signals in the cow and ewe were sufficient for maintaining CL function, and the biologically active molecules responsible for CL maintenance in these species may be very similar. Additional support for this argument

was supplied recently when antibodies produced against oTP-1 (Godkin et al., 1984) were found to cross-react with low Mr polypeptides secreted by the bovine conceptus (S.D. Helmer, W.W. Thatcher, F.W. Bazer, P.J. Hansen and R.M. Roberts, 1984 unpublished data). These observations indicate that luteal maintenance during early gestation may be achieved by analogous mechanisms in cattle and sheep.

It is interesting to note that duration of CL extension in recipient cattle (range 25-37 days) following species-specific TV transfers (Heyman et al., 1984), and ovine TV transfers (31 and 35 days; Martal et al., 1984a) is similar to luteal lifespans achieved in this study (Fig. 4-1, Table 4-1) following intrauterine treatments with pooled bovine conceptus secretory proteins in cyclic cattle (range 28-34 days).

In contrast to the observed antiluteolytic activity of conceptus secretory proteins, 5β-pregnan-3α-ol-20-one, a major, conceptus-produced steroid, did not influence CL lifespan, cycle length or decrease uterine $PGF_{2\alpha}$ production.

In conclusion, intrauterine administration of bovine conceptus secretory proteins to cyclic cattle was shown to extend CL lifespan and interestrous interval, and attenuate spontaneous uterine $PGF_{2\alpha}$ production ($PGF_{2\alpha}$ in vena cava plasma). Data presented provide strong evidence that protein signals secreted by the bovine conceptus during the

first 2 to 3 weeks of gestation are required for the establishment of pregnancy in cattle.

CHAPTER 5
INHIBITION OF ESTRADIOL-17β INDUCED UTERINE PROSTAGLANDIN-$F_{2\alpha}$ PRODUCTION BY BOVINE CONCEPTUS SECRETORY PROTEINS

Introduction

Pregnancy maintenance in cattle requires continued secretion of progesterone (P_4) by the corpus luteum (CL). To circumvent CL regression, the bovine conceptus must initiate the process of pregnancy recognition by days 15 to 17 post-estrus (Betteridge et al., 1980; Northey and French, 1980; Dalla Porta and Humblot, 1983). Previous studies in cattle suggest that there are probably several interdependent strategies by which the conceptus may initiate maintenance of the CL during early gestation. Luteotrophic and/or luteal protective substances, originating from the gravid uterine horn in cattle, were demonstrated by Del Campo and coworkers (1980). Conceptus stimulated uterine blood flow during early gestation (Ford et al., 1979; Ford and Chenault, 1981) has been proposed to increase transfer efficiency of potential luteotrophic substances from the pregnant uterus to the CL (Reynolds et al., 1983; Thatcher et al., 1984b). Conversely, endometrial and uteroovarian vascular permeability to prostaglandin (PG) $F_{2\alpha}$ is reduced during early pregnancy and may thus insure a reduction in

the delivery of luteolytic agents to the CL (Thatcher et al., 1984a,b).

Another process by which the conceptus may provide for an extension of luteal function involves attenuation of $PGF_{2\alpha}$ production by the uterine endometrium. Thatcher and coworkers (1984b) demonstrated that in vitro production of $PGF_{2\alpha}$ by day 17 pregnant endometrial explants was reduced significantly compared to production by cyclic day 17 endometrium. These data support an antiluteolytic effect of the bovine conceptus on endometrial $PGF_{2\alpha}$ synthesis.

Patterns of uterine $PGF_{2\alpha}$ production, as determined by peripheral measurements of the primary metabolite, 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM; Granstrom and Kindahl, 1982), have been characterized during the estrous cycle (Peterson et al., 1975; Kindahl et al., 1976; Betteridge et al., 1984) and early pregnancy (Kindahl et al., 1976; Betteridge et al., 1984) in cattle. Luteal regression, as determined by declining P_4 concentrations, was always associated with three or more episodic pulses of PGFM. In contrast, presence of a viable conceptus within the uterine lumen reduced or completely eliminated the PGFM episodes.

Injection of estradiol-17β (E_2) initiates an acute and reproducible increase in uterine $PGF_{2\alpha}$ production (Thatcher et al., 1984b; Chapter 3). Furthermore, uterine $PGF_{2\alpha}$ production was correlated significantly with concentrations of PGFM measured in peripheral plasma. Using the E_2-injection

model, Rico and coworkers (1981; see also: Thatcher et al., 1984b) evaluated the capacity of the uterus to synthesize and secrete $PGF_{2\alpha}$ (PGFM response) at day 18 of the estrous cycle versus days 18 and 20 of pregnancy in cows. Mean peripheral concentrations of plasma PGFM, following E_2 injection, were significantly less in pregnant versus cyclic cows. Thus the bovine conceptus appears to exert an antiluteolytic effect via modification of uterine $PGF_{2\alpha}$ production. The present study has focused on the possible antiluteolytic role of bovine conceptus secretory proteins (CSP), since intrauterine treatment with CSP to cyclic cattle (Chapter 4) and sheep (Godkin et al., 1984) resulted in extensions of CL lifespan and interestrous intervals. The objective of the present experiment was to determine if bovine conceptus secretory proteins attenuate the E_2-induced increase in uterine $PGF_{2\alpha}$ production (Thatcher et al., 1984b; Chapter 3).

Materials and Methods

Conceptus Collection and Culture

Thirty-eight Angus and crossbred beef cows were bred during estrus (day 0) and artificially inseminated approximately 12 h later. All animals were slaughtered on day 17 or 18 post-mating and reproductive tracts recovered following exsanguination. Reproductive tracts were sealed

in a plastic bag and placed on ice while being transported to the laboratory. Uterine horns were trimmed of excess tissue, ovaries and oviducts removed, and a large, curved, Rochester-Ochsner forcep applied to the anterior cervix. The anterior tip of the uterine horn ipsilateral to the CL containing ovary was cut to provide an enlarged opening. A plastic, 50 ml syringe fitted with an 18 gauge needle was used to administer two 30 ml flushes (sterile saline, warmed to 37 C) into the uterine lumen through the tip of the uncut uterine horn. Saline containing conceptuses (n=29) was collected into sterile plastic culture dishes. All conceptuses were washed immediately in 15 ml of 37 C, sterile modified (15) minimum essential medium (MEM) and transferred to sterile culture dishes containing 15 ml of MEM. Conceptuses were cultured for 30 h on a rocker platform (Bellco Glass Company, Vineland, NJ) and maintained at 37 C in a gaseous atmosphere of nitrogen:oxygen:carbon dioxide (50:45:5). Following a 30 h incubation, tissues and medium were separated by centrifugation (10,000 x g; 20 min) at 4 C. Medium (supernatant) from each incubation was collected, pooled and frozen (-20 C). Conceptus wet weights were recorded.

Preparation of Protein Solutions

Pooled medium from conceptus incubations (435 ml) was dialyzed (Spectrapore 6, 1000 Mr cutoff; Spectrum Medical Industries, Los Angeles, CA) extensively (4 L changed thrice

daily for 5 days) against 10 mM Tris-HCl buffer, pH 7.2 (TRIS). Following dialysis, culture medium was concentrated by ultrafiltration (1000 Mr cutoff filter; Amicon Corporation, Danvers, MA) to a volume of approximately 120 ml. An aliquot of medium was then used for determination of protein concentration (Lowry et al., 1951). Concentrated medium was dispensed into 2 ml aliquots (n=60) containing 0.6 mg of CSP (Treatment group). Serum proteins from a day 18 pregnant cow were dialyzed and protein concentrations determined as described for conceptus culture medium. The serum protein dialysate was appropriately diluted in TRIS and dispensed into 2 ml aliquots (n=60) containing 0.6 mg of day 18 pregnant serum proteins (Control group). Antibiotics (Polyflex, 167 mg/ml; Bristol Laboratories, Syracuse, NY) were added (200 µl) to each aliquot and frozen (-20 C) until time of intrauterine injection.

Animal Preparations

Cyclic Jersey cows (n=10) were assigned randomly to either Control or Treatment groups and prepared for surgery (Chapter 3) on day 10 of the estrous cycle. Utilizing a midventral laparotomy, the uterus and ovaries were exposed and location of the CL recorded. A sterile polyvinyl catheter (V-6; Bolab Incorporated, Lake Havasu City, AZ) was inserted and secured 45 mm into the anterior lumen of each uterine horn and exteriorized via a small flank incision. A 6 cm length of tape was folded around each catheter at the

point of exit from the body cavity and anchored to the skin with suture. Exteriorized uterine catheters were placed in a pack, consisting of an 8 x 16 cm plastic bag reinforced with tape (Ethikon; Johnson and Johnson Products Incorporated, New Brunswick, NJ), and secured to the flank with suture. Antibiotics (Polyflex, 167 mg/ml; Bristol Laboratories, Syracuse, NY) were administered on the day of surgery (10 ml I.M., 10 ml I.P. and 1 ml/each uterine horn) and one day post-surgery (10 ml I.M. and 1 ml/each uterine horn).

Intrauterine injections (n=12/cow; see above) were administered into each uterine horn at 12 h intervals (2.4 mg protein/ uterus/day) from 2000 h on day 15 to 0800 h on day 18. Blood was collected daily via jugular venipuncture from days 12 through 17 post-estrus. On day 17, all experimental cows were fitted with a jugular catheter. A jugular vein was punctured via a stainless steel, 10 cm, 12 gauge needle and a sterile polyvinyl catheter (V-9; Bolab Incorporated, Lake Havasu City, AZ) threaded through the needle barrel into the jugular vein. The needle was then withdrawn leaving approximately 40 cm of catheter in the jugular vein. The catheter was flushed with sterile, heparinized saline (Sodium Heparin, 100 units/ml; Sigma Chemical Company, St. Louis, MO), occluded with a hemostat and knotted to prevent blood flow through the catheter. Catheter remaining outside the vein was coiled and secured

to the neck with tape (Ethikon; Johnson and Johnson Products Incorporated, New Brunswick, NJ) and glue (KaMar Incorporated, Steamboat Springs, CO). Beginning at 0700 h on day 18, jugular blood samples were collected at 30 min intervals from 1 h prior to E_2 injection (3 mg in 6 ml of ethanol:0.9 % saline [1:1] via jugular vein) to 12 h post-E_2. Jugular catheters were removed immediately following the 13 h acute bleeding period. Experimental animals were fitted with estrous detection patches (Kamar Incorporated, Steamboat Springs, CO) and monitored twice daily for estrous behavior. Twice daily blood samples were drawn via jugular venipuncture from day 19 until detected estrus. Catheter placement and verification of CL regression were determined by rectal palpation at behavioral estrus in all cows.

Radioimmunoassay of Plasma Hormones

Validation of extraction and assay procedures for the P_4 radioimmunoassay used in the present study were identical to those described by Knickerbocker and coworkers (Chapter 3). Intra- and interassay coefficients of variation (CV) for 5 assays were 20.1 and 6.1%, respectively, when duplicate estimates were run in the assay for a 100 pg/500 µl (100.2 ± 6.8 pg/500 µ) plasma reference, and 12.4 and 17.9%, respectively when duplicate estimates were run for a 250 pg/500 µl (261.2 ± 17.3 pg/500 µl) plasma references. Sensitivity of the P_4 assay was 30 pg/ml.

Unextracted plasma samples (50, 100 and 200 µl) were assayed for PGFM using a polyethylene glycol radioimmunoassay system previously validated in our laboratory by Guilbault et al. (1984). The intra- and interassay CV were 13.1 and 6.1%, respectively, for a reference sample of 1000 pg/ml (1066.6 ± 35.9 pg/ml) measured in duplicate for each of 6 assays. Sensitivity of the PGFM assay was 50 pg/ml.

Statistical Analysis

Data for P_4 and PGFM concentrations in plasma, and plasma PGFM concentrations accumulated sequentially over the 12 h post-E_2 injection period (accumulated PGFM) were analyzed using the General Linear Models procedure of the Statistical Analysis System (1982) for a split-plot analysis of variance with repeated measurements over time. Analysis of variance considered variability due to treatment (CSP and Control), cow nested within treatment, time (for P_4: days 12-17; for PGFM: 30 min sequential periods for 12 h post-E_2), treatment by time, and residual. To provide estimates of time trends, plasma PGFM concentrations and accumulated PGFM values were analyzed by least squares regression analysis of variance. Differences in polynomial regressions for PGFM concentrations and accumulated PGFM time trends were tested by examining for homogeneity of regression between treatment response curves. These data were analyzed with time as a continuous, independent variable. Data

pertaining to cycle lengths and days with P_4 concentrations > 1 ng/ml were analyzed by one-way analysis of variance.

Results

Thirty-six milligrams of pooled CSP were harvested from medium following 30 h incubations of day 17 to 18 bovine conceptuses (n=29). Mean (± S.E.M.) wet weight for conceptuses was 105 ± 13 mg. A mean of 1.24 mg of CSP were produced per conceptus per 30 h and mean CSP production per mg of conceptus wet weight was 11.9 μg for a 30 h incubation.

Bovine conceptus secretory proteins administered into the uterine lumen of cyclic cattle (days 15 through 18) resulted in an attenuation ($P<0.01$) of E_2-induced uterine $PGF_{2\alpha}$ production (PGFM response) on day 18 compared to control cows which received intrauterine injections of serum proteins (Table 5-1, Fig. 5-1). Overall means for PGFM concentrations following the E_2 challenge were 74.4 ± 5.2 pg/ml for cows in the CSP treatment group and 170.6 ± 14.3 pg/ml for cows in the Control group. A treatment by time interaction also was detected ($P<0.01$; Fig. 5-1). Mean responses for the Control group exhibited two distinct phases during which PGFM concentrations were transiently elevated above baeline (Fig. 5-1). The initial rise occurred between 2 to 3.5 h with peak PGFM concentrations at approximately

Table 5-1. Progesterone (P_4) concentrations prior to E_2 injection (3 mg) and prostaglandin, luteal lifespan and interestrous interval following E_2 in cyclic cows treated with serum proteins (CONTROL) or conceptus secretory proteins (CSP)

C O N T R O L

Cow	P_4 (ng/ml)[a]	Accumulated[b] PGFM (pg)	Luteal Lifespan (days)	Interestrous Interval (days)
J164	9.6±1.3	5063	20.0	22.0
J85	10.2±1.5	4720	19.0	23.0
J190	11.7±1.0	2402	20.0	21.0
J240	7.3±1.3	2184	21.0	24.0
J160	10.0±0.6	708	21.0	21.5
Overall Mean ± S.E.	9.7 ±1.3	3016 ± 821	20.2 ± 0.4	22.3 ± 0.5

C S P

Cow	P_4 (ng/ml)	Accumulated PGFM (pg)	Luteal Lifespan (days)	Interestrous Interval (days)
J97	3.5±0.2	1505	20.0	21.0
J193	4.9±0.7	1408	20.0	22.0
J216	7.9±1.5	102	22.5	25.5
J181	10.6±0.5	33	25.0	26.0
J155	9.9±0.5	0	22.5	24.5
Overall Mean ± S.E.	7.4[ns] ±1.4	610** ± 347	22.0[ns] ± 0.9	23.8[ns] ± 1.0

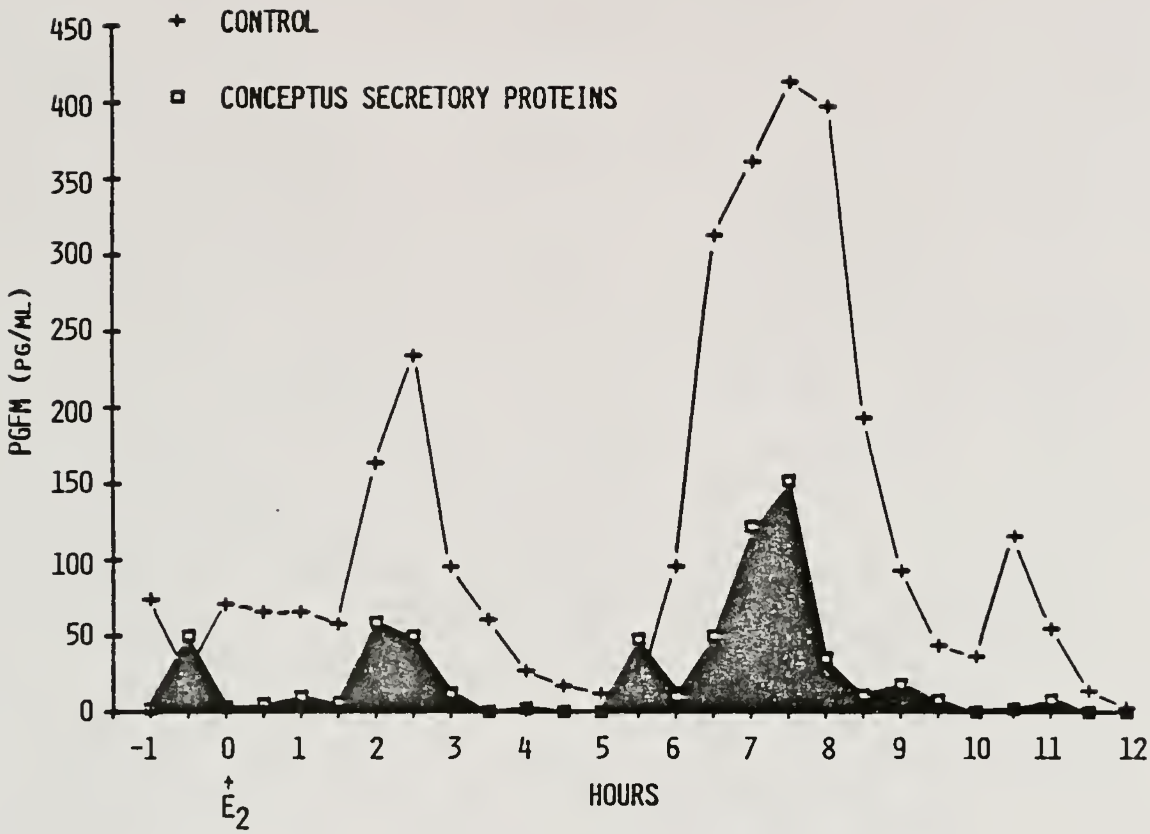
[a] Mean plasma progesterone calculated from days 12-17, prior to E_2.

[b] Total 15-keto-13,14-dihydro-$PGF_{2\alpha}$ accumulated over the 12 h period post-E_2.

[ns] nonsignificant difference; P>0.1.

** P<0.01; (CSP<CONTROL).

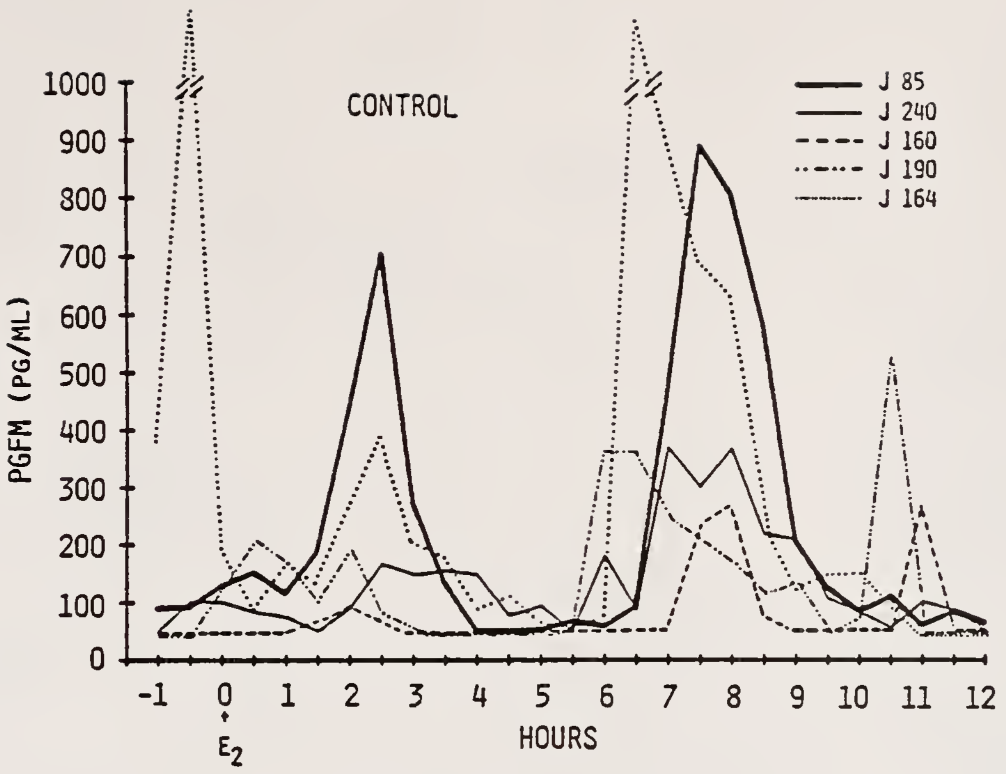
Figure 5-1. Least squares means of plasma 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM) concentrations in response to an estradiol-17β (E_2) challenge (3 mg; I.V.) in cyclic Jersey cows receiving intrauterine infusions (days 15.5-18) of serum proteins from a day 18 pregnant cow (control; n=5) or conceptus secretory proteins (n=5).

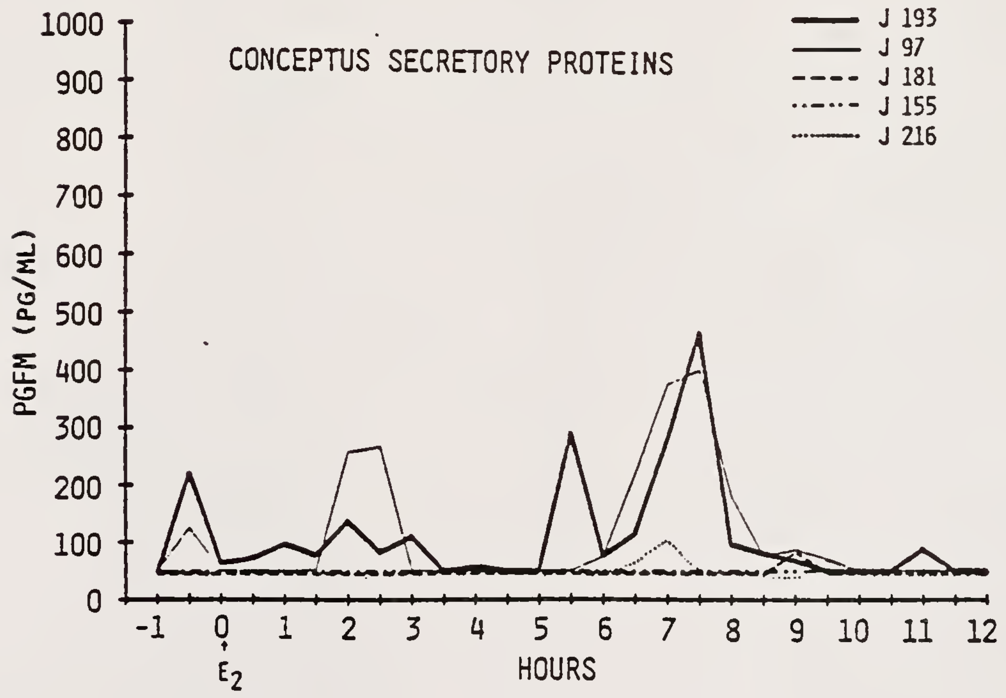

CONTROL
CONCEPTUS SECRETORY PROTEINS
PGFM (PG/ML)
450
400
350
300
250
200
150
100
50
0
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
E_2
HOURS

2.5 h. A second and more substantial peak was initiated by approximately 6 to 6.5 h, peaked at 7.5 h, and returned to baseline concentrations by 8.5 to 9.5 h post-E_2. Mean PGFM response in CSP-treated cows was approximately 20% of that observed for the control group as determined by areas under the two PGFM peaks and mean accumulated PGFM concentrations following E_2 injection (Table 5-1). Individual PGFM responses for each cow are depicted in figure 5-2. All cows in the Control group responded to exogenous E_2 by having elevations in plasma PGFM concentrations. In one animal (J164), a spontaneous pulse of PGFM (2725 pg/ml) was detected prior to the E_2 challenge which suggested that luteal regression had already been initiated. Concentrations of plasma P_4 had declined from 6.4 ng/ml on day 17 to 2.1 ng/ml on the day of E_2 injection (day 18). Uterine $PGF_{2\alpha}$ production was most dramatic in this cow as demonstrated by the induced PGFM peak at 6.5 h (1225 pg/ml).

In contrast, three of five cows treated with CSP failed to respond to injection of E_2. The remaining two cows exhibited PGFM responses similar to low responders (n=3) in the control group. Interestingly, mean P_4 concentrations from days 12 to 17 of the estrous cycle (Table 5-1) were considerably lower in the two cows which did not exhibit a clear antiluteolytic effect of CSP post-E_2 injection (3.5 ± 0.2, 4.9 ± 0.7 ng/ml versus 7.9 ± 1.5, 9.9 ± 0.5, 10.6 ± 0.5 ng/ml). Cows in the Control group had P_4 concentrations

Figure 5-2. Individual cow plasma concentrations of 15-keto-13,14,-dihydro-$PGF_{2\alpha}$ (PGFM) in response to an estradiol-17β (E_2 challenge (3 mg; I.V.) in cyclic Jersey cows receiving intrauterine infusions (days 15.5-18) of serum proteins from a day 18 pregnant cow (control) or conceptus secretory proteins.


CONTROL
J 85
J 240
J 160
J 190
J 164
PGFM (PG/ML)
1000
900
800
700
600
500
400
300
200
100
0
-1 0 1 2 3 4 5 6 7 8 9 10 11 12
E_2
HOURS


CONCEPTUS SECRETORY PROTEINS
J 193
J 97
J 181
J 155
J 216
PGFM (PG/ML)
1000
900
800
700
600
500
400
300
200
100
0
-1 0 1 2 3 4 5 6 7 8 9 10 11 12
E_2
HOURS

which fluctuated between 5 and 14 ng/ml ($\bar{X} \pm$ S.E., 9.7 ± 1.3 ng/ml) during the same period. The PGFM responses were evaluated further by accumulating PGFM concentrations over the 12 h period following E_2 injection (Table 5-1). On the average, cows in the Control group responded to an E_2 challenge by accumulating approximately five times more PGFM ($P<0.01$) than cows which received intrauterine treatments of CSP (Table 5-1). This point is illustrated (Figure 5-2) by comparing profiles for PGFM over the 12 h post-E_2 period in Control and CSP groups.

No differences ($P>0.25$) were detected in CL lifespan (days with P_4 > 1 ng/ml) or estrous cycle length responses between the two experimental groups (Table 5-1). However, the three cows in the CSP group which accumulated the least PGFM tended to have extended CL maintenance and longer interestrous intervals.

Rectal palpation of the reproductive tracts at estrus confirmed catheter placements within the tip of each uterine horn. No uterine or ovarian abnormalities were apparent. Ovarian palpation per rectum and analysis of plasma P_4 concentrations verified regression of the CL which was observed at surgery.

Discussion

Bovine conceptus secretory proteins significantly attenuated uterine production of $PGF_{2\alpha}$, as determined by plasma PGFM concentrations, following injection of a luteolytic dose of E_2 to cyclic cows. These data are consistent with responses described previously for cyclic cattle treated with bovine CSP (Chapter 4) in which a significant reduction in vena cava plasma concentrations of $PGF_{2\alpha}$ was associated with extended CL function and interestrous interval. Collectively, these reports indicate that the local antiluteolytic influence of the bovine conceptus, in utero (Kindahl et al., 1976; Betteridge et al., 1984) results from an interaction between biologically active conceptus-derived proteins and the uterine endometrium (Thatcher et al., 1984a).

However, mechanisms by which bovine CSP reduce endometrial production of $PGF_{2\alpha}$ are not known. Data in sheep, as in cattle, demonstrated that intrauterine administration of conceptus derived secretory proteins to cyclic ewes resulted in prolonged CL maintenance and interestrous interval (Godkin et al., 1984), and attenuated E_2-stimulated rise in plasma PGFM concentrations (Fincher et al., 1984). Furthermore, ovine trophoblast protein-1 (oTP-1), the major conceptus produced protein during maternal recognition of pregnancy in sheep, extended luteal function when introduced

into the uterine lumen (Godkin et al., 1984a), bound specifically to endometrial receptors and stimulated secretion of specific endometrial proteins in vitro (Godkin et al., 1984b). Although no specific functions have been attributed to these endometrial proteins in sheep, there are reports which demonstrate the presence of inhibitors to prostaglandin synthesis in bovine endometrium (Wlodawer et al., 1976) and caruncular (Shemesh et al., 1984) tissues. Additionally, Thatcher and coworkers (1984b) demonstrated a reduction in de novo $PGF_{2\alpha}$ synthesis by bovine endometrial explants at day 17 of pregnancy compared to day 17 of the estrous cycle. Arachidonic acid supplementation to endometrial incubations increased $PGF_{2\alpha}$ production in both pregnant and cyclic tissues. However, $PGF_{2\alpha}$ production was less for pregnant endometrium. It is possible that bovine conceptus secretory proteins activate endometrial biosynthesis of inhibitors to prostaglandin production resulting in selective inhibition of uterine $PGF_{2\alpha}$ production (Thatcher et al., 1984b; Chapter 4) while permitting biosynthesis of prostaglandins by the developing conceptus (Shemesh et al., 1979; Lewis et al., 1982). Additionally, conceptus-induced uterine prostaglandin inhibitors may modify the amounts and/or classes of prostaglandins synthesized by the developing conceptus and endometrium during early gestation (Lewis and Waterman,

1983; Lewis, 1984), as well as placentome tissues during later gestation (Shemesh et al., 1981; 1984).

In the present study, the maternal endocrine milieu may influence uterine responsiveness to conceptus signals during the critical period of pregnancy recognition. Intrauterine treatments with CSP, in two of five cows, did not prevent the E_2-stimulated rise in plasma PGFM concentrations. These animals had 50 to 60% lower P_4 concentrations (4.2 ± 0.3 ng/ml) during the luteal phase of the estrous cycle (days 12 through 17) compared to P_4 concentrations exhibited by three cows (9.5 ± 0.7 ng/ml) in which CSP completely inhibited the E_2-induced PGFM response. Progesterone is known to regulate uterine physiological development in numerous species. The rate and extent of uterine and conceptus development (synchrony) may be altered by variations in length of P_4 exposure and P_4 concentrations during early gestation (Lawson and Cahill, 1983; Lawson et al., 1983). Thus, inferior CL quality, as reflected by low plasma P_4 concentrations, may preclude appropriate uterine development and result in an inadequate maternal response to conceptus signals.

Estrogen stimulation of uterine $PGF_{2\alpha}$ production (Chapter 3) is thought to involve uterine protein synthesis, since the luteolytic effect of estrogen may be prevented by inhibiting uterine DNA dependent RNA synthesis with actinomycin D (French and Casida, 1973). Elevated estrogen

production by ovarian follicles may initiate luteolysis in cattle, as destruction of follicles during the luteal phase of the estrous cycle prolongs CL function (Villa-Godoy et al., 1981). Additionally, Huslig and coworkers (1979) reported cyclic changes in concentrations of uterine cyclooxygenase in ewes which occurred simultaneously with changes in uterine $PGF_{2\alpha}$ secretion. They suggested that exposure of the P_4 primed uterus to estrogen was responsible for synthesis of this rate limiting enzyme. Estrogen is also believed to induce the formation of endometrial oxytocin receptors during the estrous cycle (Roberts et al., 1976; McCracken et al., 1981). Exogenous oxytocin stimulates uterine $PGF_{2\alpha}$ production in cyclic cattle (Newcomb et al., 1977; Milvae and Hansel, 1980) and causes premature CL regression (Armstrong and Hansel, 1959). Oxytocin is believed to augment uterine $PGF_{2\alpha}$ production as the estrogen:P_4 ratio increases following initiation of luteolysis and thus ensures rapid and complete CL regression (Wathes, 1984). In sheep, endometrial oxytocin receptor concentrations are significantly reduced at day 16 of pregnancy versus day 16 of the estrous cycle (McCracken et al., 1981) and the oxytocin induced rise in plasma PGFM concentrations are attenuated in E_2-primed cyclic ewes following intrauterine CSP administration (Fincher et al., 1984). Therefore, bovine and ovine CSP may regulate synthesis or availability of steroid receptors responsible for oxytocin receptor

induction, or CSP may modify the formation or availability of endometrial oxytocin receptors directly to prevent luteolysis.

A biphasic PGFM response (at 2.5 and 7.5 h) to E_2 was observed in all control cows and two of five CSP treatment cows. Timing of the first PGFM peak coincided with maximal uterine blood flow response following stimulation by exogenous E_2 in cattle (Roman-Ponce et al., 1978; Chapter 3), and probably represents a washing out of residual prostaglandins from an active uterus. The second and more substantial peak of PGFM corresponded to the period, post-E_2, when uterine $PGF_{2\alpha}$ production is activated (Chapter 3).

Lastly, bovine CSP have been shown previously to extend CL function and interestrous interval in cyclic cows when administered into the uterine lumen at 12 h intervals from days 15 to 21 post-estrus (Chapter 4). However, mean CL lifespan and interestrous intervals in the present study did not differ significantly between experimental groups. The difference in response to bovine CSP in the first (Chapter 4) and present study are probably due to the shorter period of CSP treatments (4 versus 7 days), and the post-CSP challenge with a luteolytic dose of E_2 (3 mg) in the present study. There was a tendency, however, for extended CL function and cycle length in CSP treatment cows which accumulated the least PGFM (Table 5-1).

In conclusion, these data support a role for conceptus secretory proteins in suppression of uterine $PGF_{2\alpha}$ production during the period of pregnancy recognition in cattle.

CHAPTER 6
GENERAL DISCUSSION

Advances in our understanding of mechanisms controlling luteal maintenance during early pregnancy have been considerable during the last decade. Characterization of conceptus products and their biosynthetic patterns have provided an important foundation from which many of the exciting findings reported herein stem. The bovine conceptus produces an array of biologically active steroids, prostaglandins and proteins during early pregnancy which are involved in conceptus developmental processes and alterations in maternal physiological events directed toward pregnancy maintenance (see: Chapter 1). Elevated endocrine activity by bovine conceptus tissues occurs concomitantly with rapid conceptus elongation and maternal recognition of pregnancy. Maternal recognition of pregnancy represents the first critical period during early gestation when conceptus signals must be synthesized and received subsequently by the maternal system such that cyclical events regulating corpus luteum (CL) regression are circumvented. Currently, evidence suggests that several conceptus-mediated strategies are involved to this end. These strategies may be classified broadly as 1) luteotropic (stimulation of luteal

P_4 production); 2) luteal protective (block luteolytic effects of $PGF_{2\alpha}$ at the level of the CL); and 3) antiluteolytic (attenuation of uterine $PGF_{2\alpha}$ production).

Antiluteolytic effects of conceptus products were evaluated in two experiments (Chapters 4 and 5). Data from these studies demonstrated that bovine conceptus-derived protein signals extended CL function and interestrous intervals, and attenuated spontaneous and E_2-induced uterine $PGF_{2\alpha}$ production. Durations of CL and interestrous interval extension following intrauterine administration (days 15.5 to 21) of bovine CSP were 30.3 and 33.4 days, respectively, compared to 22.3 and 23.5 days, respectively for control cows (Chapter 4). Bovine CSP influence on the uterus remained effective for one to two weeks (range: 7 to 13 days) following the last intrauterine injection. This response differs from responses reported previously to intrauterine administration of PGE_2 (Chenault, 1983; Gimenez and Henricks, 1983; Reynolds et al., 1983). Prostaglandin-E_2 effects on CL maintenance are short-lived in cyclic cattle suggesting that this substance will not prevent production and transfer of uterine luteolytic substances to the CL. Available data support luteotropic (Marsh, 1970) and/or luteal protective (Henderson et al., 1977; Reynolds et al., 1981) roles for PGE_2 during early pregnancy. At present, precise mechanism(s) by which bovine CSP attenuate uterine $PGF_{2\alpha}$ production is unknown. However, in ovine

endometrium, estrogen (Findlay et al., 1982) and oxytocin (Roberts et al., 1976; McCracken et al., 1984) receptor concentrations are reduced during early pregnancy. Likewise, exogenous E_2- (cow: Knickerbocker et al., 1984; Chapter 5; sheep: Fincher et al., 1984) and oxytocin- (sheep: Fincher et al., 1984) induced uterine $PGF_{2\alpha}$ production are attenuated following intrauterine CSP administration. Conversely, decline in endometrial estrogen and oxytocin receptors may result from maintenance of endometrial sensitivity to P_4 (viz., P_4 receptors). Progesterone inhibits replenishment of E_2 receptors and estrogen dependent responses (see: Chapter 1) such as induction of endometrial oxytocin receptor production (McCracken et al., 1984). Maintenance or increased endometrial P_4 receptor concentrations during early pregnancy may be influenced by CSP, however, modifications in steroid receptor populations are generally thought to be steroid regulated (see: Chapter 1). Other conceptus products, such as estrogens (Chapter 2) or catecholestrogens (hydroxylated estrogen metabolites) may mediate endometrial P_4 sensitivity. Alternatively, effects of antiluteolytic bovine CSP may be directed at reducing synthesis or activity of arachidonic acid metabolic enzymes within the uterine endometrium. This effect may occur directly or indirectly via stimulation of uterine inhibitors of prostaglandin synthesis. The extended antiluteolytic effect of bovine CSP

detected after cessation of intrauterine treatments (Chapter 4) support the hypothesis that CSP induce endometrial production of inhibitors to prostaglandin synthesis. An extended antiluteolytic effect of the conceptus was also observed in endometrial explant cultures of day 17 pregnant cattle (Thatcher et al., 1984b). Synthesis of specific endometrial proteins, in vitro, were induced by an ovine conceptus protein, oTP-1 (Godkin et al., 1984a). Lastly, Wlodawer et al. (1976) reported that bovine endometrium contains an inhibitor of prostaglandin biosynthesis. Mediation of conceptus antiluteolytic effects through induced endometrial production of prostaglandin synthesis inhibitors is an attractive concept for the following reasons: 1) uterine $PGF_{2\alpha}$ biosynthesis may be prevented without inhibiting prostaglandin production by the developing conceptus; 2) ratio of PGE_2:$PGF_{2\alpha}$ within the uterine lumen may be elevated as a result of continued conceptus prostaglandin production and decreased uterine $PGF_{2\alpha}$ production; and 3) conceptus estrogen production (Chapter 2) may stimulate uterine blood flow and facilitate movement of luteotropic (PGE_2, PGI_2)/luteal protective (PGE_2) prostaglandins to the CL without stimulating uterine $PGF_{2\alpha}$ biosynthesis. Similarly, conceptus induced endometrial inhibitors may regulate biosynthesis of products from the lipoxygenase pathway during early pregnancy. Milvae and coworkers (Milvae and Hansel, 1980, 1983; Milvae

et al., 1985) have demonstrated (in vivo and in vitro) that PGI_2 is luteotropic in cattle and may be essential for normal luteal development and continued P_4 biosynthesis. Prostacyclin biosynthesis is inhibited by products of the lipoxygenase pathway, e.g., 5-HETE. Furthermore, intrauterine administration (days 14 to 18) of a lipoxygenase pathway blocker, NDGA, to cyclic cattle extended luteal function to day 25. Thus, 5-HETE and other lipoxygenase products may be involved in normal luteolytic events. Inhibition of lipoxygenase products during early pregnancy may therefore facilitate PGI_2 biosynthesis (uterus, conceptus, CL) and luteotropic actions on the CL.

In contrast to the clear antiluteolytic effect of conceptus secretory proteins, a major conceptus steroid product, 5β-pregnan-3α-ol-20-one (5β-P; Chapter 2), had no effect on luteal function or cycle length when administered into the uterine lumen of cyclic cattle (Chapter 4). In fact, total spontaneous $PGF_{2\alpha}$ measured in vena cava samples were greater ($P<.01$) in cows treated with 5β-P than homologous serum proteins (Control). No adequate explanation for this result can be offered at this time. However, recent data (Thatcher et al., 1984b) have demonstrated decreased endometrial and uteroovarian vascular permeability to $PGF_{2\alpha}$ in day 17 pregnant cows. If intrauterine administration of 5β-P reduced tissue permeability to $PGF_{2\alpha}$ without preventing or attenuating

uterine $PGF_{2\alpha}$ production, an accumulation of $PGF_{2\alpha}$ might be expected to occur within the uterine lumen. Such elevated uterine luminal concentrations of $PGF_{2\alpha}$ may translate ultimately into greater uterine venous concentrations of $PGF_{2\alpha}$ as maximum tissue capacity is reached. Alternatively, progestational activity of 5β-P may be sufficient to stimulate endometrial accumulation of lipid stores and prostaglandin precursors, thereby resulting in greater uterine capacity to synthesize $PGF_{2\alpha}$. Other putative roles for the 5β-reduced steroids are discussed in Chapter 2. Further research directed toward understanding conceptus and endometrial steroid function is warranted.

Collectively, research described herein support the use of peripheral PGFM concentrations as an index of uterine $PGF_{2\alpha}$ synthetic activity. The use of the E_2-challenge scheme (characterized in Chapter 3) proved an effective tool for evaluating uterine capacity to synthesize $PGF_{2\alpha}$ following intrauterine treatments. Spontaneous $PGF_{2\alpha}$ episodes and E_2-induced uterine $PGF_{2\alpha}$ production were dampened significantly by intrauterine administration of bovine CSP, but not 5β-pregnan-3α-ol-20-one, a major conceptus steroid product. Bovine CSP did not influence CL production of P_4 suggesting that CSP act locally at the uterine level.

APPENDIX A
MANUFACTURE OF SEPHADEX LH20 COLUMNS

1. Prepare a slurry of Sephadex LH20 beads in freshly distilled solvents. Solvent mixture should be identical to that which is employed during steroid separation.
2. Allow beads to swell for 12 to 24 hours at room temperature in a sealed container.
3. Degas slurry under a vacuum of approximately 25 p.s.i. for 15 to 20 minutes before pouring column.
4. Glass columns (35 x 1.5 cm) are filled with the solvent mixture and stopcock moved to the open position.
5. Sephadex slurry is pipetted into the column until the desired column height is reached. At this point, approximately 50 ml of the solvent mixture is run over the column to aid in packing. Do not let column run dry.
6. Turn column stopcock off, add solvent mixture to approximately 25 cm on the glass column, and seal column top.
7. Columns are allowed to settle and pack for 12 to 24 hours before use.

APPENDIX B
STEROID ELUTION BY GAS/LIQUID CHROMATOGRAPHY

Steroid[a]	minutes	R_f/P_4[b]
5β-androstane-3β,17α-diol	5.85	.140
4-pregnen-3β-ol-20-one	6.0	.144
5β-androstane-3β,17β-diol	6.25	.150
5α-androstane-3α,17α-diol	6.5	.156
5β-androstane-3α,17α-diol	6.6	.158
5α-androstane-3α,17β-diol	6.75	.162
5β-androstane-3α,17β-diol	6.75	.162
4-androstene-3β,17β-diol	6.75	.162
5α-androstane-3β,17α-diol	7.1	.170
5α-androstane-3β,17β-diol	7.25	.174
5β-pregnane-3β,20β-diol	8.5	.204
5β-pregnane-3β,20α-diol	9.1	.218
5β-pregnane-3α,20β-diol	9.4	.225
5β-androstan-3β-ol-17-one	9.6	.230
5α-pregnane-3α,20α-diol	9.8	.235
5α-pregnane-3β,20β-diol	10.1	.242
5α-pregnane-3α,20β-diol	10.25	.246
5β-pregnane-3α,20α-diol	10.25	.246
5α-androstan-3α-ol-17-one	10.25	.246
5α-pregnane-3β,20α-diol	10.9	.261
5β-androstan-3α-ol-17-one	11.0	.263
5α-androstan-3β-ol-17-one	11.75	.281
5β-androstan-17α-ol-3-one	12.1	.290
5β-pregnan-3β-ol-20-one	12.5	.299
5β-androstan-17β-ol-3-one	12.75	.305
5α-androstan-17α-ol-3-one	12.9	.309
5α-pregnan-3α-ol-20-one	13.5	.323
5α-androstan-17β-ol-3-one	13.6	.326
5β-pregnan-3α-ol-20-one	13.75	.329
5α-pregnan-3β-ol-20-one	15.0	.359
4-androsten-17α-ol-3-one	19.25	.461
4-androsten-17β-ol-3-one	20.1	.481
5β-androstane-3,17-dione	20.75	.497
5α-androstane-3,17-dione	22.25	.533

Steroid[a]	minutes	R_f/P_4[b]
5β-pregnane-3,20-dione	26.1	.625
4-pregnen-20β-ol-3-one	27.4	.656
4-pregnen-3β,20β-diol	27.75	.665
5β-pregnane-3,20-dione	28.1	.673
4-pregnen-3,17-dione	31.0	.743
4-androstene-3,17-dione	32.4	.776
4-pregnene-3,20-dione	41.75	1.000
4-pregnene-17α-ol-3,20-dione	61.0	1.461

Note: Column specifications: 3% trifluropropyl-silicone (SP-2401) on Supelcoport 100/120 mesh (Supelcoport Inc., Bellefonte, PA)
Detector: flame ionization
Column temperature: 240 C
Injector temperature: 265 C
Detector temperature: 260 C
N_2 pressure: 26.5 p.s.i.
Gas flow rates (ml/min): H_2 (20); air (300)

[a] Gift from National Research Council, Steroid Reference Collection, London, England.

[b] Retention time relative to 4-pregnene-3,20-dione.

APPENDIX C
MASS CALCULATIONS FOR ESTROGENS

<u>STEP 1</u>. Convert sample cpm to dpm.

$$\frac{\text{Sample cpm}^{a}}{\text{Counting Efficiency during liquid scintillation}} = \text{Sample dpm}$$

<u>STEP 2</u>. Calculate dpm/pg estrogen.

A. Specific Activity (Ci/mmole) of 1,2,6,7-[^{3}H](N)-P_4 substrate

B. 2.22×10^{12} dpm = 1 Ci

C. Molecular weights:

Estrone	270.3
Estradiol-17β	272.3
Estriol	288.3

Calculations:[b] $\frac{A \times B}{C}$ = dpm/mg estrogen

then divided by 10^9 = dpm/pg estrogen

<u>STEP 3</u>. Calculate pg of estrogen in sample.

Calculation: $\frac{\text{Sample dpm}}{\text{dpm/pg estrogen}}$ = pg estrogen

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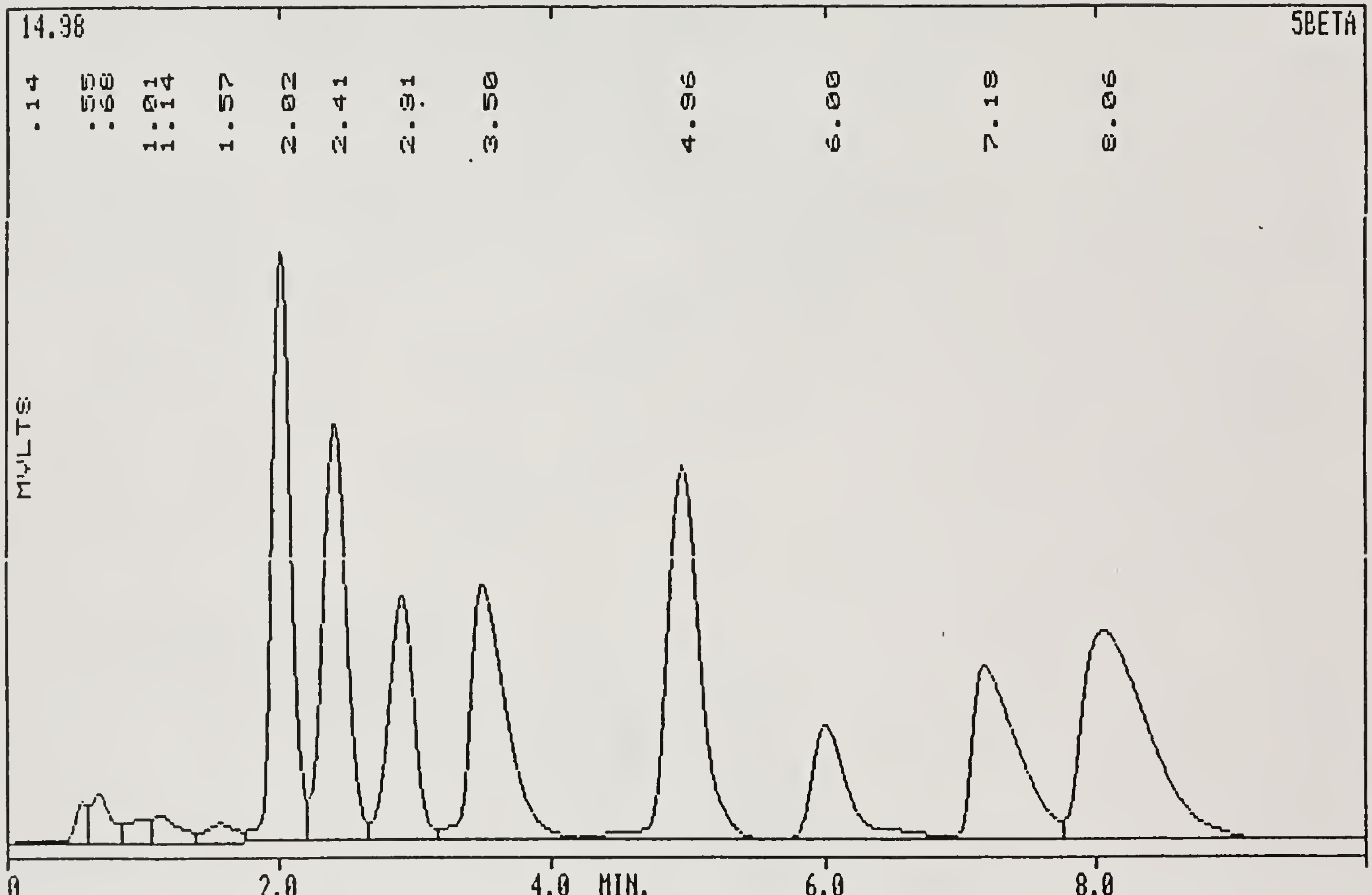
[a] Corrected for background cpm and [^{14}C] spillover.

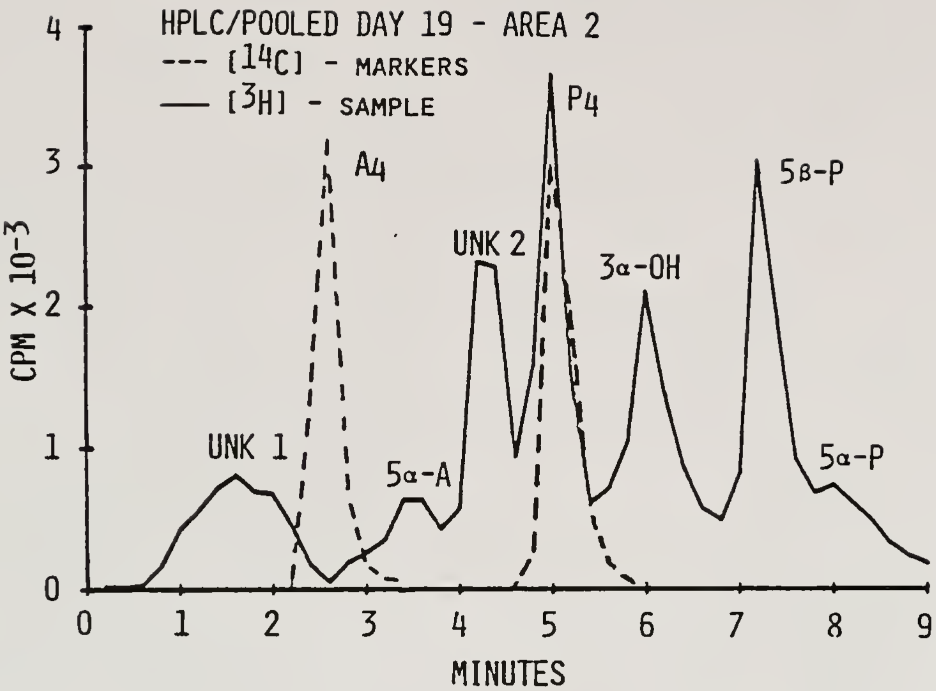
[b] These calculations assume that the specific activity of estrogen products equal the specific activity of tritiated P_4 substrate. This assumption is justifed by two points. First, placement of tritiated hydrogens on the 1,2,6, and 7 positions of P_4 are in an α-configuration (Dr. Mayo Cabell, New England Nuclear, Boston, MA; personal communication). Second, enzymatic steps in aromatization involve the loss of A-ring hydrogens at the 1β or 2β sites (Fishman, 1982). Thus, loss of 1α, 2α, 6α or 7α tritiums during aromatization are not warranted.

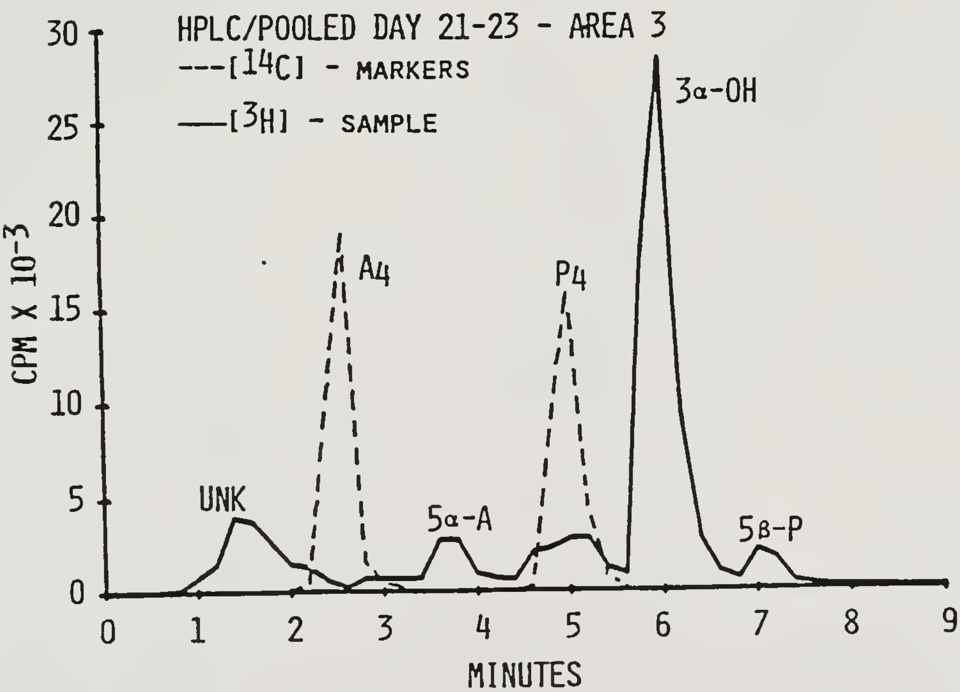
APPENDIX D
ELUTION OF RADIOINERT STEROID STANDARDS ON HPLC (ACETONITRILE:WATER, 54:46)



Testosterone (2.02 min.), Androstenedione (2.41 min.), 5β-Androstanedione (2.91 min.), 5α-Androstanedione (3.50 min.), Progesterone (4.96 min.), 5β-pregnan-3α-ol-20-one (6.00 min.), 5β-pregnanedione (7.18 min.), 5α-pregnanedione (8.06 min.).

APPENDIX E
ELUTION OF [^{3}H]-CONCEPTUS METABOLITES AND [^{14}C]-MARKERS ON HPLC (ACETONITRILE:WATER, 54:46)





Abbreviations: UNK (unknown), A_4 (androstenedione), 5α-A (5α-androstanedione, P_4 (progesterone), 3α-OH (5β-pregnan-3α-ol-20-one), 5β-P (5β-pregnanedione), 5α-P (5α-pregnanedione).

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BIOGRAPHICAL SKETCH

Jeffrey John Knickerbocker, eldest of four children born to John Vearon Knickerbocker and Janice Danforth, was born in Eatontown, New Jersey, on April 17, 1955. During his first 15 years, he acquired a taste for travel as his parents made several work-related moves, including a memorable two years in Frankfurt, West Germany. In 1973, he graduated from Neptune Senior High School, Neptune, New Jersey, and attended the University of Delaware, home of the "Fighting Blue Hens," until 1977 when he graduated with a Bachelor of Science in animal science. He began his graduate study in the fall of 1977 at Clemson University, South Carolina, under the guidance of Dr. Joseph F. Dickey in the Department of Dairy Science. On August 5, 1978, he was married to Margaret 'Holly' Hollywood Dougherty of Claymont, Delaware. In 1982, he completed his degree requirements for the Master of Science in food and animal industries, Clemson University, South Carolina.

During the fall of 1979, he and his wife arrived in Gainesville, Florida, where he began a program of research under the direction of Dr. William W. Thatcher in the Department of Dairy Science, University of Florida, Gainesville. His training in reproductive biology at the

University of Florida may be said to have been a truly fertile experience. During this period, two children, Lauren (June 23, 1980) and Daniel John (December 11, 1983) were born to him and Holly.

Upon completion of the Doctor of Philosophy degree, the author will further his training in the laboratory of Dr. Gordon D. Niswender, Colorado State University, Fort Collins, as a post-doctoral fellow.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William W. Thatcher

William W. Thatcher, Chairman
Professor of Animal Science and Dairy Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Fuller W. Bazer

Fuller W. Bazer
Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Michael J. Fields

Michael J. Fields
Associate Professor of Animal Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Professor of Biochemistry and Molecular Biology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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